

Fluorescent Gold Nanorod Probes for the Detection of Cancer mRNA Biomarkers

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Declaration

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Abstract

Cancer is a leading cause of death worldwide and high mortality rates are due to diagnosis at advanced disease stages, where the success of treatment is reduced. Current cancer diagnostic techniques rely on anatomical imaging or invasive tumour biopsies that lack the sensitivity to detect early disease stages. Circulating cancer biomarkers, including circulating tumour cells and cell free DNAs and RNAs offer the opportunity to non-invasively detect cancer signatures at early disease states. Current investigations into liquid biopsies for the detection of circulating tumour cells and cell free RNAs lack sensitivity, specificity and involve complex sample processing.

Therefore, there is a need for an early cancer detection method to improve treatment success and patient outcomes. As cancer cells display different genetics from healthy cells, genetic biomarkers have become of great interest for cancer detection. RNA biomarkers are of particular interest, as they can be detected in the serum of cancer patients even at early disease stages. Here, the synthesis of fluorescent gold nanorod probes for the detection of cancer mRNA biomarkers in liquid biopsies will be reported.

The nanoprobe consists of gold nanorods functionalised with fluorophore labelled hairpin DNA that is targeted against a cancer mRNA biomarker sequence. Due to the optical properties of gold nanorods, the fluorophore is quenched by energy transfer in the closed hairpin DNA conformation and fluorescence is only generated upon target mRNA hybridisation. This provides a high signal to background ratio due to the surface plasmon enhanced energy transfer in the hairpin DNA conformation and surface plasmon enhanced fluorescence following target hybridisation.

Steady state and time resolved fluorescence spectroscopy of the nanoprobe incubated with non-target and target DNA has demonstrated the nanoprobe binding specificity. Flow cytometry of cell lines incubated with nanoprobe has demonstrated the nanoprobe is capable of specifically detecting target mRNA within individual target mRNA expressing cells and the fluorescence of the

nanoprobe is proportional to target mRNA expression levels. Nanoprobe uptake into cells and target binding specificity in cancer cells has been demonstrated through fluorescence confocal microscopy and fluorescence lifetime imaging microscopy, with both the fluorescence emission and fluorescence lifetime of the nanoprobe increasing after incubation with target mRNA expressing cells. Liquid biopsies from cancer patients have also been investigated which demonstrated the presence of a fluorescence population in cancer positive samples following nanoprobe incubation, demonstrating the ability of nanoprobe to detect circulating tumour cells. Furthermore, the ability of nanoprobe to detect target mRNA in exosomes extracted from cancer cell lines was demonstrated, providing the potential for nanoprobe to detect cell free circulating mRNAs within liquid biopsies. These investigations have demonstrates the capability of nanoprobe for detecting cancer mRNA biomarkers in liquid biopsies and the potential clinical applications of nanoprobe in cancer detection.

While nanoprobe optimisation was initially focused on one cancer biomarker, C-MYC, nanoprobe have been designed against several other mRNA cancer biomarkers of interest, including HER1, HER2, SOX2, as well as wildtype and mutational P53. Successful synthesis of these nanoprobe show the potential for multiplexed biomarker detection. Molecular sensing and imaging of mRNA biomarkers demonstrated in this work provides the potential for a sensitive, non-invasive and early cancer detection method from liquid biopsies.

Conference Proceedings

G. Craig, T. Hupp, D.J.S. Birch and Y. Chen. “Nanoprobes for mRNA Imaging” Poster Presentation, 2016, TransMed Conference, Edinburgh.

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Abbreviations

BP – base pair

cDNA – complementary DNA

CTAB – cetyltrimethylammonium bromide

CTC – circulating tumour cell

ctDNA – circulating tumour DNA

CV – coefficient of variation

Cy5 – cyanine 5

DDT – dodecanethiol

DNA – deoxyribonucleic acid

FLIM – fluorescence lifetime imaging microscopy

FRET – Förster resonance energy transfer

FSC – forward scatter

GNR – gold nanorod

HER1 – human epidermal growth factor receptor 1

HER2 – human epiderman growth factor receptor 2

hpDNA – hairpin deoxyribonucleic acid

MHA – mercaptohexanoic acid

mRNA – messenger ribonucleic acid

NGS – next generation sequencing

NTA – nanoparticle tracking analysis

P53 – tumour protein P53

PCR – polymerase chain reaction

qRT-PCR – quantitative real time polymerase chain reaction

RNA – ribonucleic acid

SDS – sodium dodecyl sulfate

SET – surface energy transfer

smFISH – single molecule sensitivity fluorescent in situ hybridization (smFISH)

SOX2 – Sry-related HMG box 2

SPR – surface plasmon resonance

SSC – side scatter

St dev – standard deviation

TCEP – tris(2-carboxyethyl)phosphine hydrochloride

TCSPC – time correlated single photon counting

T_m – melting temperature

TPL – two photon luminescence

UV – ultraviolet

Contents

Declaration.....	i
Acknowledgements	ii
Abstract.....	iv
Conference Proceedings	vi
1. Introduction	1
1.1. Gold nanorod properties.....	1
1.2. Circulating biomarkers for early cancer diagnostics	6
1.3. Current methods to detect circulating cancer biomarkers	10
1.4. Fluorescent mRNA Nanoprobes	14
1.5. Gold nanorod probes for fluorescence-based detection of mRNA biomarkers	20
1.6. Conclusion	24
2. Experimental Methodology	25
2.1. Gold nanorod extinction spectra by UV-Vis spectroscopy	25
2.2. Steady state fluorescence spectroscopy	26
2.3. Time resolved fluorescence spectroscopy	28
2.4. Flow cytometry.....	30
2.5. Confocal fluorescence imaging	34
2.6. Fluorescence lifetime imaging microscopy	35
3. Optimising gold nanoprobe synthesis and investigating nanoprobe opening dynamics by fluorescence spectroscopy.....	38
3.1. Introduction	38
3.2. Sample preparation and characterisation	44
3.2.1. Materials.....	44

3.2.2. Gold nanorod synthesis	44
3.2.3. Gold nanorod ligand exchange	45
3.2.4. Gold nanorod DNA functionalisation.....	45
3.2.5. Steady state fluorescence spectroscopy	46
3.2.6. Time resolved fluorescence spectroscopy.....	47
3.3. Results	47
3.3.1. Investigating nanorods stability after synthesis and ligand exchange	47
3.3.2. Optimisation of DNA loading onto GNRs through varying the pH of salting buffer	53
3.3.3. Investigating specificity of nanoprobe to target DNA through fluorescence spectroscopy	56
3.3.4. Hybridisation of nanoprobe with targets are both time and target concentration dependent.....	58
3.3.5. Fluorescence lifetime recovery of the nanoprobe after cDNA incubation	60
3.4. Conclusions	65
4. Investigating the uptake and fluorescence of C-MYC mRNA nanoprobe in individual cells using flow cytometry	68
4.1. Introduction	68
4.2. Experimental Methods.....	72
4.2.1. Materials.....	72
4.2.2. Sample preparation	72
4.2.3. Flow cytometry measurement	73
4.2.4. qRT-PCR	73
4.3. Results	75
4.3.1. Investigating gold nanorod uptake in cell lines	75

4.3.2. Investigating nanoprobe synthesis conditions on fluorescence in cell lines	78
4.3.3. Investigating nanoprobe sensitivity to C-MYC expression levels in cell lines	83
4.3.4. Investigating the dependence of nanoprobe fluorescence on hpDNA dynamics.....	85
4.3.5. Detecting cancer cells in co-culture.....	87
4.4. Conclusions	91
5. Designing Nanoprobe against multiple mRNA biomarkers and mutational P53.	93
5.1. Introduction	93
5.1.1. Probe design	93
5.1.2. Selection of cancer biomarkers	95
5.2. Sample preparation.....	99
5.2.1. Materials.....	99
5.2.2. Steady state fluorescence spectroscopy	99
5.2.3. Flow cytometry.....	99
5.3. Results	100
5.3.1. Nanoprobe design	100
5.3.2. Validation of new nanoprobe designs by fluorescence spectroscopy and flow cytometry	105
5.3.3. Designing nanoprobe against a P53 mutation biomarker.....	110
5.4. Conclusions	123
6. Demonstrating the internalisation of nanoprobe and target mRNA specific fluorescence in individual cells by confocal fluorescence microscopy & fluorescence lifetime imaging microscopy	125
6.1. Introduction	125

6.2. Sample preparation.....	131
6.2.1. Materials.....	131
6.2.2. Preparing cells on coverslips.....	132
6.2.3. Confocal imaging	132
6.2.4. Fluorescence lifetime imaging.....	132
6.3. Results	133
6.3.1. Visualisation of nanoprobe uptake in cells using fluorescence confocal microscopy	133
6.3.2. Detection of biomarker mRNA in cells by fluorescence lifetime imaging microscopy	139
6.3.3. FLIM image analysis of nanoprobe in cells.....	144
6.3.4. Ability to differentiate quenched and unquenched cy5 within the nanoprobe by FLIM.....	155
6.4. Conclusions	162
7. Investigating the clinical potential of nanoprobe in liquid biopsy	164
7.1. Introduction	164
7.2. Sample preparation.....	167
7.2.1. Materials.....	167
7.2.2. Clinical sample preparation and measurement by flow cytometry.....	167
7.3. Results	168
7.3.1. Investigating the clinical applications of C-MYC nanoprobe in pleural effusion liquid biopsies.....	168
7.3.2. Investigating the clinical applications of SOX2 nanoprobe in pleural effusion liquid biopsies.....	179
7.3.3. Investigating the clinical applications of nanoprobe in blood sample liquid biopsies.....	190

7.4. Conclusions	203
8. Investigating the ability of nanoprobe to detect target mRNA in exosomes	205
8.1. Introduction	205
8.2. Sample preparation.....	207
8.2.1. Materials.....	207
8.2.2. Exosome extraction	207
8.2.3. Measuring exosome concentration by NanoSight.....	207
8.2.4. Steady state fluorescence spectroscopy	208
8.2.5. Time resolved fluorescence spectroscopy.....	208
8.3. Results	208
8.3.1. Investigating the ability of nanoprobe to detect exosomes isolated from cell lines by fluorescence spectroscopy.....	208
8.4. Conclusions	220
9. Summary and Future work	222
9.1. Summary	222
9.2. Future work.....	225
9.2.1. An investigation into the mechanisms of GNR uptake	225
9.2.2. Multiplex flow cytometry measurements	227
9.3.3. Continuation of clinical pleural effusion samples.....	228
9.3. Conclusion	228
10. References	230

Figures

Figure 1. Localised surface plasmon resonance of gold nanorods.	2
Figure 2. Principles of FRET.	5
Figure 3. Circulating tumour cells and circulating tumour DNA are biomarkers that can be detected in the blood.	8
Figure 4. Jablonski diagram of fluorescence.	15
Figure 5. Molecular beacons for RNA sensing.	16
Figure 6. Nanoflares for mRNA detection	18
Figure 7. Schematic of the gold nanoprobe design.	22
Figure 8. A typical set up of a UV-Vis spectrometer.	26
Figure 9. Schematic of steady state fluorescence spectroscopy.	27
Figure 10. Schematic of TCSPC by time resolved fluorescence spectroscopy.	29
Figure 11. Principles of flow cytometry.	32
Figure 12. Schematic of the MoFlo XDP detection pathways.	33
Figure 13. Fluorescence imaging using a confocal microscope.	35
Figure 14. A typical configuration of fluorescence lifetime imaging microscopy.	37
Figure 15. Process of gold nanorod synthesis, ligand exchange and DNA functionalisation.	42
Figure 16. The extinction spectra of GNRs is controlled during synthesis.	49
Figure 17. The extinction spectra & zeta potential before & after ligand exchange confirm stability of GNR structure and a successful ligand exchange.	51
Figure 18. SEM images and size distribution analysis show nanorod shape stability and uniformity.	52
Figure 19. Fluorescence emission change after target DNA incubation is dependent on the pH of salting buffer.	55
Figure 20. Differential fluorescence emission intensity demonstrates the specificity of nanoprobe binding.	57
Figure 21. Nanoprobe opening is dependent on the concentration of DNA target and the incubation time.	59
Figure 22. Fluorescence lifetime recovery of cy5 after target DNA incubation.	63

Figure 23. Flow cytometry demonstrates a time and dose dependent uptake of gold nanorods in cell lines.	77
Figure 24. Investigating salting condition on the fluorescence intensity of the nanoprobe in cells.	82
Figure 25. Detecting differential C-MYC mRNA expression in cell lines by qRT-PCR and flow cytometry of the C-MYC nanoprobe.	84
Figure 26. Nanoprobe fluorescence is dependent on hpDNA opening dynamics.	86
Figure 27. C-MYC nanoprobe can selectively detect C-MYC expressing cancer cells in co-culture.	90
Figure 28. The regulation network of P53 in response to cellular stress signals.	97
Figure 29. Fluorescence spectroscopy of nanoprobe before and after target DNA incubation.	106
Figure 30. Nanoprobes exhibit fluorescence intensity in target mRNA expressing cell lines.	109
Figure 31. P53 wildtype and mutant nanoprobe designs.	111
Figure 32. Fluorescence intensity of P53 wildtype and P53 mutation design 1 nanoprobe after complimentary or one nucleotide mismatch DNA incubation.	114
Figure 33. Fluorescence intensity of P53 wildtype and P53 mutation design 2 nanoprobe after complimentary or one nucleotide mismatch DNA incubation.	116
Figure 34. Flow cytometry of wildtype and mutant P53 nanoprobe in P53 wildtype expressing cells demonstrates differential fluorescence.	118
Figure 35. Fate of nanoparticles following endocytosis.	126
Figure 36. Fluorescence imaging of gold nanorods and nanoprobe in Hek & PC3 cells.	134
Figure 37. Fluorescence analysis of individual nanoprobe in Hek & PC3 cells.	136
Figure 38. Z-stack of nanoprobe in PC3 cells.	138
Figure 39. Fluorescence lifetime imaging of gold nanorods and nanoprobe in Hek & PC3 cells.	140

Figure 40. Lifetime values of c-myc nanoprobe in Hek & PC3 cells.	143
Figure 41. The phasor plot vector space.	145
Figure 42. Fluorescence lifetime analysis of GNRs and nanoprobe in cells.	148
Figure 43. Fluorescence lifetime analysis of only the GNR and nanoprobe contribution in cells.	151
Figure 44. Fluorescence lifetime analysis of C-MYC nanoprobe areas in in Hek cells.	153
Figure 45. Fluorescence lifetime analysis of C-MYC nanoprobe areas in PC3 cells.	154
Figure 46. Fluorescence emission of the C-MYC nanoprobe with nanoprobe alone and nanoprobe treated with cDNA.	156
Figure 47. Fluorescence lifetime imaging of nanoprobe and nanoprobe-cDNA in Hek and PC3 cells.	157
Figure 48. FLIM analysis of open and closed nanoprobe in Hek and PC3 cells.	160
Figure 49. The percentage of fluorescence events detected in pleural effusion samples from cancer patients following nanoprobe incubation.	171
Figure 50. Flow cytometry analysis of a benign pleural effusion liquid biopsy following incubation with a c-myc nanoprobe.	175
Figure 51. Flow cytometry analysis of a metastatic breast cancer pleural effusion liquid biopsy following incubation with a c-myc nanoprobe.	177
Figure 52. Flow cytometry analysis of a metastatic lung cancer pleural effusion liquid biopsy following incubation with a c-myc nanoprobe.	179
Figure 53. Flow cytometry analysis of a metastatic breast cancer pleural effusion liquid biopsy following incubation with a SOX2 nanoprobe.	184
Figure 54. Flow cytometry analysis of a metastatic cancer pleural effusion liquid biopsy following incubation with a SOX2 nanoprobe.	186
Figure 55. Flow cytometry analysis of a metastatic lung cancer pleural effusion liquid biopsy following incubation with a SOX2 nanoprobe.	188
Figure 56. The percentage of fluorescence events detected in blood samples from cancer patients following nanoprobe incubation.	193

Figure 57. Flow cytometry analysis of a metastatic breast cancer blood sample liquid biopsy following incubation with a C-MYC nanoprobe.	196
Figure 58. Flow cytometry analysis of a metastatic breast cancer blood sample liquid biopsy following incubation with a C-MYC nanoprobe.	197
Figure 59. Flow cytometry analysis of a metastatic cervical cancer blood sample liquid biopsy following incubation with a C-MYC nanoprobe.	198
Figure 60. Evaluation of the range of fluorescence events detected after C-MYC and SOX-2 nanoprobe incubation in liquid biopsies.	200
Figure 61. Size distribution of exosomes and gold nanorods by NanoSight.	211
Figure 62. Fluorescence emission spectroscopy of exosomes extracted from Hek and PC3 cells before and after nanoprobe incubation.	214
Figure 63. Time resolved fluorescence spectroscopy of C-MYC nanoprobe after incubation with exosomes extracted from Hek and PC3 cells.	217
Figure 64. Peak fluorescence emission spectroscopy of the nanoprobe incubated with a PC3 exosome dilution.	220
Figure 65. Calcein leakage assay for nanoparticle escape from endosomes.	226

Tables

Table 1. Advantages and disadvantages of current methods of mRNA biomarker detection.	19
Table 2. Fluorescence lifetime 3-exponential fitting analysis.	64
Table 3. Nanoprobe design sequences, melting temperatures and structure for each mRNA biomarker.	104
Table 4. The selectivity coefficient of P53 nanoprobes design 1 in solution phase.	120
Table 5. The selectivity coefficient of P53 nanoprobes design 2 in solution phase.	120
Table 6. The selectivity coefficient of P53 nanoprobes in Flo-1 cells by flow cytometry.	121
Table 7. Fluorescence lifetime values for individual pixel FLIM analysis.	141
Table 8. Fluorescence lifetime values from individual pixels from FLIM of open and closed hpDNA in Hek and PC3 cells.	158
Table 9. The percentage of fluorescence events detected by flow cytometry in pleural effusion patient samples following C-MYC nanoprobe incubation.	170
Table 10. The percentage of fluorescence events detected by flow cytometry in pleural effusion patient samples following SOX2 nanoprobe incubation.	180
Table 11. Comparing the fluorescent signal intensity of effusion samples from individual patients that were incubated with both C-MYC and SOX-2 nanoprobes.....	190
Table 12. The percentage of fluorescence events detected by flow cytometry in blood samples from cancer patients following nanoprobe incubation.....	192
Table 13. The concentration of exosomes and gold nanorods measured by NanoSight.	212
Table 14. Fluorescence lifetime decay fitting analysis for C-MYC nanoprobe before and after incubation with exosomes from Hek and PC3 cells.	218

1. Introduction

1.1. Gold nanorod properties

Nanotechnology is a new and exciting interdisciplinary research field that combines engineering, physics, chemistry, biology and medicine. It has a huge potential for medical applications in the diagnosis and personalised treatments of human diseases. It is a rapidly expanding area of research area, with large centres being developed such as the National Nanotechnology Initiative in USA, which has received over \$22 billion in funding since it was established in 2001 (1). Cancer nanotechnology is the fastest expanding area in the biomedical applications, with research being carried out for the use of nanoparticles for in vitro and in vivo imaging (2), diagnostics (3), therapy (4) and drug delivery (5).

Nanoparticles range from 1-100 nm in size and their properties are dependent on their composition, shape and size. They can be made from a range of materials such as silver, gold, iron and carbon and produced in a number of shapes such as spheres, rods, tubes, triangles and stars (6). Gold nanoparticles are of interest in biological sensing and imaging due to their unique optical properties. In particular, gold nanorods (GNRs) are of particular interest due to the unique properties they possess at the nanoscale.

Gold nanorods are a relatively recent development and have gained a lot of interest since the early 2000's. The optical properties of GNRs are determined by their aspect ratio, i.e., the ratio between the rod's length and its width. The aspect ratio of GNRs can be tuned during synthesis, providing different absorption and scattering properties, resulting from surface plasmon resonance (SPR), which will be discussed. GNRs can undergo two-photon luminescence, providing a high contrast agent within cells (7) and can be utilised for fluorescence lifetime imaging microscopy (FLIM) (8), as well as distance dependant energy transfer and fluorescence enhancement of chromophores (9). GNRs are also biocompatible, as they can be synthesised to have low toxicity to cells and they have the ability to be

conjugated to biomolecules, with efficient cell uptake and high stability within cells (10). All of these properties make GNRs an attractive agent for biological sensing and imaging.

The SPR is an attractive property of GNRs (11) (Figure 1). This is an optical phenomenon that occurs due to the nanoparticles large surface area and the confinement of its surface plasmons, which are delocalised electron oscillations. The excitation of free electrons within the conduction band by incoming photons creates an enhanced local electromagnetic field, with oscillations of the free electron cloud around the metal core of the particle. This surface plasmon resonance provides an enhancement in the optical properties of absorption and scattering (12). Gold nanorods display two surface plasmon absorbance peaks, a tunable peak from between 600 nm > 1000 nm that corresponds to the surface plasmon mode along the long axis (longitudinal mode) and a peak around 520 nm that corresponds to the surface plasmon mode along the short axis (transverse mode).

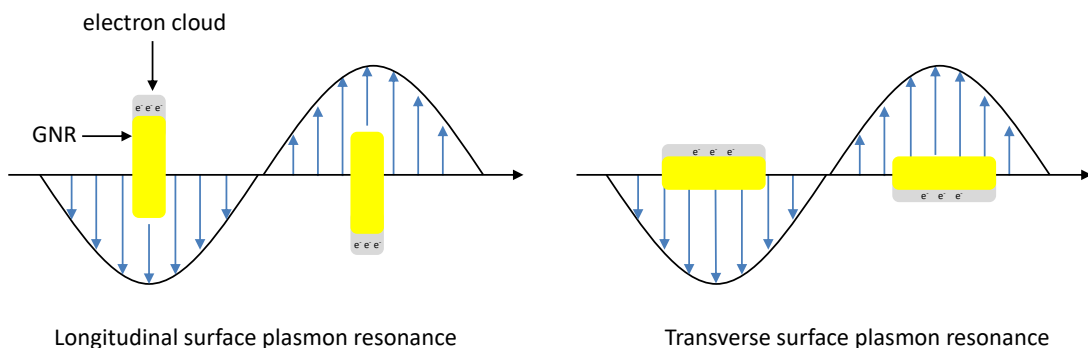


Figure 1. Localised surface plasmon resonance of gold nanorods.

Gold nanorods display oscillations of free electron clouds around the longitudinal and transverse axes.

These strong absorption properties make GNRs a highly efficient fluorescence quencher through energy transfer (13). Förster resonant energy transfer (FRET) is energy transfer that occurs between a donor chromophore that is excited to an

acceptor, which absorbs this energy through nonradiative dipole–dipole coupling. The extent of energy transfer depends on the overlap of the donors emission and the acceptors absorption peaks (14) as well as the physical distance between the donor and acceptor (Figure 2). The efficiency of FRET is inversely proportional to the 6th order of the distance between the donor and acceptor, making FRET sensitive to small changes in distance (15). The GNR absorption peak can be tuned during synthesis to overlap with the emission of the target fluorophore (16). The typical distance range between an acceptor and donor is 1-8nm in FRET, however the use of metallic nanoparticles as acceptors increases this distance up to ~20nm due to the highly efficient quenching abilities, as detailed below.

The energy transfer from a dye to metal can be described through the surface energy transfer (SET) model, where the electromagnetic field of the donor dipole interacts with the free conduction electrons of the acceptor metal nanoparticles (17). In surface energy transfer, the transfer rate $k_{\tau}(r)$ is given by Equation 1. Here, d is the separation distance between the donor fluorophore and the acceptor nanoparticle and τ_D is the lifetime of the donor and d_0 is the distance at which SET efficiency decreases to 50%.

$$k_{\tau}(r) = \frac{1}{\tau_D} \left(\frac{d_0}{d} \right)^4$$

Equation 1.

As well as quenching abilities, gold on the nanoscale also has fluorescence enhancement abilities. Metal enhanced fluorescence is facilitated by the local surface plasmon resonance, which is an enhancement in the local electromagnetic field. This results in an enhancement in the absorption and emission and modifications in the quantum yield of the fluorophore, with the fluorescence enhancement factor of a fluorophore increasing as the longitudinal plasmon wavelength of GNRs is tuned close to the excitation and emission wavelength (18). A fluorophore positioned near the GNR surface can undergo coupling with the

electromagnetic field, which results in an enhanced fluorescence emission. Reports have shown that the fluorescence intensities can increase 10-fold or more when the fluorophore is at an optimal distance from the gold nanoparticle surface (19).

Fluorophores within biosensors must be photostable and have a high intensity to be detected above background fluorescence. Metallic nanoparticles provide a way to enhance the optical properties of fluorophores. This is due to plasmon coupling between the metal and the fluorophore, which results in an increased quantum yield and increased photostability. Metal enhanced fluorescence is reported to occur when the fluorophore is positioned from 5 to 90 nm away from the gold surface (20). Fluorescence quenching occurs below 20 nm in distance, therefore the distance between the gold and fluorophore is an important factor in a fluorescent nano-sensor design.

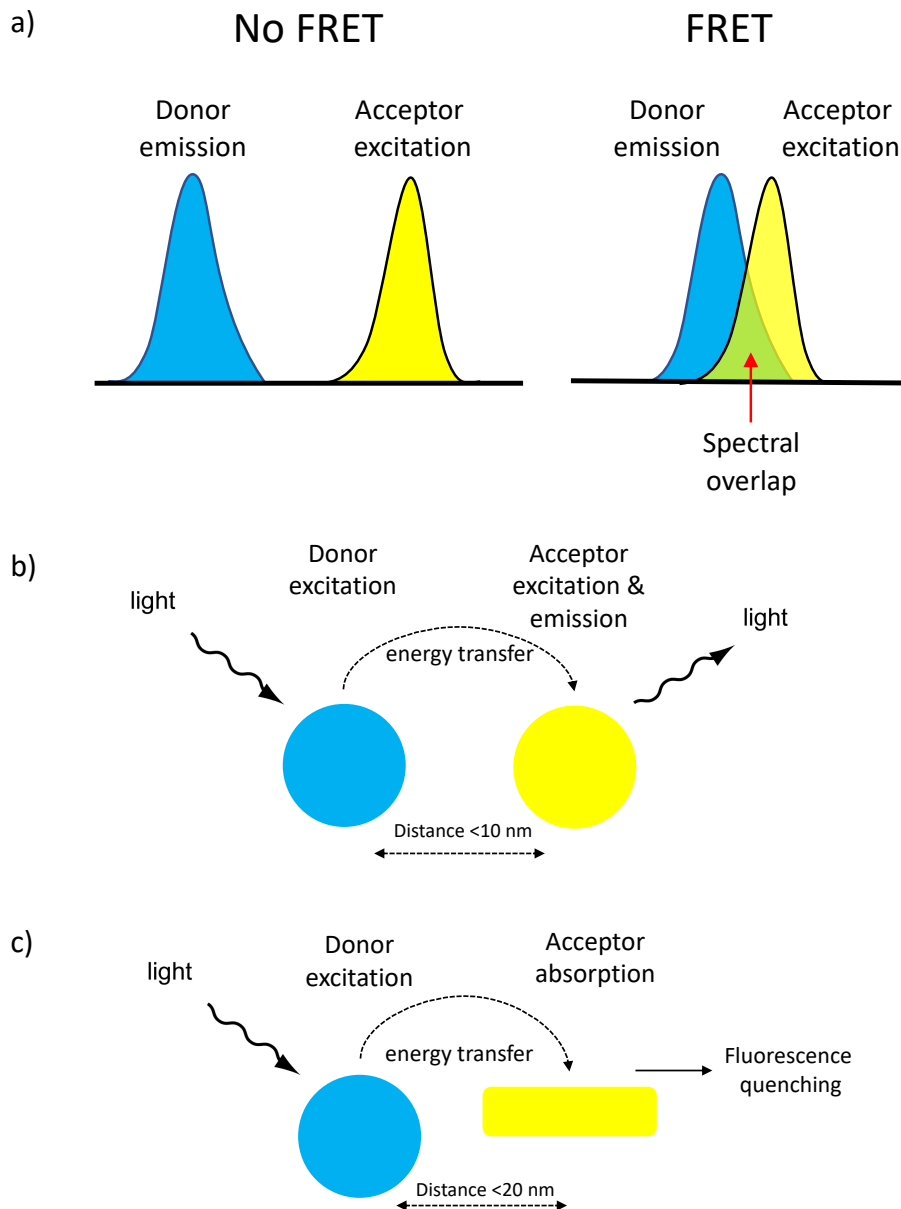


Figure 2. Principles of FRET.

a) FRET depends on the extent of spectral overlap between the donor emission. b) FRET also depends on the distance between the donor and acceptor pair. When FRET occurs between two fluorophores that are less than 10 nm in proximity, the acceptor fluorophore is excited by the donor emission. c) When FRET occurs between a fluorophore donor and a gold nanorod acceptor, the absorption properties of the GNR quenches the fluorescence of the fluorophore. The distance over which FRET can occur is increased when gold nanoparticles are used as the acceptor.

1.2. Circulating biomarkers for early cancer diagnostics

Cancer is a major cause of death worldwide, causing over 14 million new cases and over 8.2 million deaths a year (21). A lack of low-cost and rapid diagnosis techniques results in the majority of cancer diagnoses occurring at an advanced disease stage. When cancers are diagnosed at advanced stages, the success of treatment and patient survival is greatly reduced and late diagnostics are largely responsible for high mortality rates (22). Therefore, there is the need for more efficient cancer screening methods to detect the disease at early stages and improve patient outcome. Cancer cells have a very different gene expression from healthy cells, therefore genetic biomarkers have become of great interest (23). The ability to detect cancer specific biomarkers would be a valuable clinical diagnostic tool.

During the diagnostic procedure, cancers are commonly categorised by a staging system that grades the severity of the disease. This ranges from stage 0, where there are abnormal cells present and may become cancer, to stage 1 where the cancer is small and contained within the organ of origin, stage 2 where the tumour has become larger and has migrated into nearby lymph nodes, stage 3 where the cancer has started to spread to surrounding tissues and stage 4 where the cancer has spread to other organs, known as metastasis (24).

The staging at diagnosis is very important for patient outcome. For example, non-small cell lung cancer is responsible for the most cancer related deaths worldwide. The one-year survival rate for non-small cell lung cancer patients is 83% when diagnosed at stage 1, but this rapidly drops to 17% when diagnosed at stage 4 (25). The five-year survival rate when diagnosed at stage 4 is less than 2%. With over 65% of lung cancer patients being diagnosed at an advanced or metastatic stage (26), there is a real need for a more sensitive, accurate and rapid method for cancer screening and diagnostics.

Biomarkers are defined as a characteristic that can be identified and measured as an indicator of a specific biological process. An ideal biomarker is a signature that is present in the disease state of interest, that is not present in healthy cells and this

can be expressed as an overexpression or a specific mutation. Furthermore, a reliable biomarker must be stably expressed in all disease stages, to allow for early disease diagnostics, and be able to be isolated or targeted.

There is a great interest in identifying biomarkers that can be used as specific cancer identifiers. Currently, there are very limited biomarkers in clinical use for cancer diagnosis. Diagnosis techniques rely on anatomical imaging, including X-ray, CT and MRI scans, after the patient presents with symptoms associated with the cancer type. While these imaging techniques provide information on tumour size and location, they are time consuming, expensive, do not provide information on tumour physiology and are not sensitive to detect early stages of the disease (27). A tumour biopsy is a standard diagnostic technique that removes a sample from one area of the tumour for analysis. Tissue biopsy is a risky procedure and is invasive and painful for the patient. In some cases, a biopsy is not possible due to the location of the tumour. The procedure is also very expensive, and it is not practical to perform repeatedly to assess the tumours response to treatment.

Furthermore, tissue biopsies provide limited information due to tumour heterogeneity. Tumours are complex organs that are made up of many different subpopulations, which are genetically distinct (28). Tumour biopsies are often imaged for a disease specific mutation but if these are missed this can lead to inappropriate treatment decisions which can lead to treatment resistance and disease progression (28). Therefore, sampling one area may miss important tumour signatures and morphology and may not be accurate in determining which treatment the tumour will respond to. A non-invasive cancer biomarker detection procedure would be beneficial to patients, as it would allow diagnostics to occur at early disease stages and may provide an opportunity for a more comprehensive tumour analysis.

There is currently no single biomarker for cancer detection and challenges in biomarker detection and validation include the heterogeneity of the disease and the multiple mutation pathways that occur within cancers. In every cancer there are

multiple genetic drivers that lead to the growth of a tumour and these genetic changes are not present in healthy tissues. These changes can be amplifications or mutations in key genes that drive the progression of the disease. A method that can non-invasively detect these genetic changes, the cancer biomarkers, would provide valuable information about the molecular makeup of individual tumours. The detection of circulating cancer biomarkers offers such an opportunity.

Circulating biomarkers are an attractive cancer diagnostic technique as they provide a non-invasive method to identify cancer signatures. There are two types of cancer-derived biomarkers in blood: circulating tumour cells (CTC), that are shed from primary or metastatic tumours, and cell-free circulating tumour DNA and RNAs (ctRNA) (29) (Figure 3).

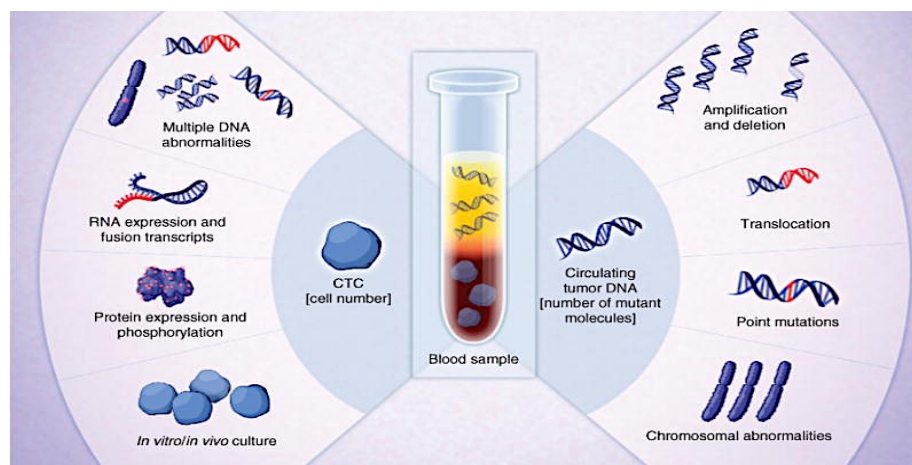


Figure 3. Circulating tumour cells and circulating tumour DNA are biomarkers that can be detected in the blood.

Cancer biomarkers that can be detected in the blood include circulating tumour cells that are shed from primary or metastatic tumours and result in tumour sites at new body regions. Circulating tumour DNA are short fragments of DNA and RNA that exist in the bloodstream and are produced from dead tumour cells. Both biomarkers carry information that can detect the presence of cancers before the presentation of symptoms. Image was adapted with permission from the American Association for Cancer Research, Copyright © 2014.

Circulating tumour cells are whole cancer cells that are shed from primary or metastatic tumours and are released into the circulatory system. CTCs were first observed in 1869, when they were found in the blood of a metastatic cancer patient (30). CTCs are thought to be the mechanism by which cancer spreads in the body, as these cells can either be identified and destroyed by the immune system or they can enter new sites in the body where they seed and start a new tumour growth.

The second type of circulating genetic biomarkers is cell free circulating tumour DNA and RNA (ctDNA and ctRNA). These are short fragments of genetic material that are released into the bloodstream and it is not fully understood how this process occurs. It is believed some cell free material is created through cancer cell death, as tumours are very unstable and display a high level of cell death. There is also evidence that DNA and RNA can be actively secreted from tumour cells and are transported in the blood through exosomes, which may be a method of cell communication (31). These exosome vesicles protect the nucleotides from nucleases in the blood and reduce immune cell recognition.

Cell free DNA and RNA were first discovered in the blood in 1948 (32) but it was not until 1994 that it was found to carry tumour-specific mutations in the serum of cancer patients (33). It was then discovered that amount of circulating DNA was higher in cancer patients when compared to healthy individuals (34), opening the potential for this material to be used for disease identification and characterisation. The concentration of circulating DNA in healthy individuals has been reported to be around 1 ng/mL, while in cancer patients this can range from 5 – 1500 ng/mL (35).

The ctDNA concentration has been found to be independent of the CTC concentration, suggesting independent mechanisms for entering the circulation. ctDNA has also been reported to correlate to the patients response to chemotherapy, suggesting these biomarkers could also be used as a marker of treatment response (36). It was also confirmed that circulating DNA in cancer patients originates from the tumour site, as it displays the same mutation found in the tumour mass (37)(38). Therefore, the discovery and characterisation of CTCs

and ctDNA has opened the possibility of liquid biopsies for disease screening and diagnostics.

Messenger RNA (mRNA) is the intermediate stage between the DNA gene and the cellular protein and represents the cells transcriptional state. Cancer cells have a very different protein expression from normal cells, therefore there is the opportunity to exploit this to specifically detect the presence of malignant cells. It has been reported that circulating RNAs are upregulated in the blood of cancer patients compared to healthy individuals (39). It has also been demonstrated that cell free mRNA can be detected in the serum of cancer patients from early disease stages (40), including lung (41), breast (42) and colorectal cancers (43). Therefore, mRNA biomarkers are of particular interest as they can be detected in the serum of cancer patients from early disease stages, providing the potential for a non-invasive and early cancer detection method through molecular sensing.

1.3. Current methods to detect circulating cancer biomarkers

There is a huge amount of interest in the biomedical diagnostic field to develop a technique that can detect specific oligonucleotide biomarkers. Specifically, there is interest in developing liquid biopsies from blood, as this would provide a rapid, non-invasive testing procedure that is possible to routinely carry out and repeat before, during and after treatment to detect disease and monitor patient response to treatment. A rapid diagnostic test would also reduce the time and cost associated with current tissue biopsy diagnostic techniques (44).

There have been several methods developed to detect cancer biomarkers in liquid biopsies. The challenge is to develop a sensing technique that is sensitive enough to detect rare cancer biomarkers. For CTCs, it has been reported that they exist in a range of 1-10 per ml of blood, in a background of millions of white blood cells and billions of red blood cells (45). Several methods have been investigated to isolate and characterise whole CTCs from blood as a diagnostic technique.

CellSearch is a CTC isolation method that identifies cancer cells based on specific cell surface markers. This technology uses iron nanoparticles coated in an antibody that recognises a cancer cell surface marker, EpCAM. However, this method has been criticised for its low capture rate and variable detection rates, limiting its reliability in specifically identifying cancer cells (46) (47).

Other methods of CTC capture include size-based filtration. This takes advantage of the relatively large size of CTCs, compared with other hematopoietic cells. This procedure uses membrane filter cylindrical 8 µm pores, which capture and fix CTCs to the membrane. However, using the size filtration method has drawbacks, as fixation of the cells onto the membrane limits the downstream analysis and it has been found to result in non-specific capture of cell debris (48).

Advances in DNA sequencing have also made it possible to identify cancer ctDNAs. Genome sequencing has led to the identification of cancer mutation hotspots, which has uncovered the most prevalent biomarkers in cancer subtypes. One method that has been developed to detect ctDNA is the cancer personalized profiling by deep sequencing (CAPP-Seq) (49). This was first developed for the detection of non-small cell lung cancer biomarkers. They found that the levels of ctDNA detected were highly correlated with the tumour volume. This method was capable of detecting ctDNA in 100% of patients with stage II to stage IV and in 50% of patients with stage I. Although DNA sequencing is sensitive in detecting ctDNA, it is still a very expensive and labour-intensive method to perform whole exome sequencing from each patient. Furthermore, whole genome amplification has been reported to be susceptible to technical errors, which can result in loss of DNA coverage and can introduce false positives and negatives (50)(51).

There have also been wide a range of methods developed for the analysis of CTCs after capture. In one investigation, human blood was spiked with cancer cells from the SK-MEL-28 melanoma cell line, to mimic CTCs. The cancer cells were then isolated using a commercial CTC isolation kit, which was found to have a recovery rate of 59.2%. The captured cells were then analysed by next generation sequencing

(NGS) to identify cancer specific gene expression (52). While this provides a detailed analysis, it requires complex CTC capture methods, that does not result in the capture of all CTCs and it requires expensive sequencing and analysis techniques.

Another technique that has been widely used for cancer biomarker detection is polymerase chain reaction (PCR). This is a technique that uses short primer recognition sequences to bind and amplify specific target DNA sequences. Multiplex digital PCR has been used to detect circulating tumour DNA. It has been shown to be capable of detecting 7 common mutations in the KRAS oncogene in the plasma from colorectal cancer patients (53). This method is capable of detecting 1 mutation DNA sequence in a background of 10,000 wildtype copies, providing a limit of detection of 0.01%. PCR has also been shown to be able to detect DNA within CTCs. A study into CTCs in the blood of lung cancer patients demonstrated that PCR was capable of detecting the expression of 3 cancer biomarkers. Sensitivity of individual biomarker detection in cancer patient blood ranged from 60% to 72%. When these biomarkers were used in combination, the sensitivity of CTC detection increased to 80% (54).

PCR provides a high sensitivity for sequence specific DNA recognition. In order for PCR to be used to detect RNA levels, reverse transcription is performed before the PCR amplification (RT-PCR) to convert RNA to complementary DNA strands, which can then be recognised by the PCR primers. Reports have confirmed that circulating mRNAs can be detected through RT-PCR. Shih et al. studied circulating RNAs in the blood of lung cancer patients (55). RNAs were extracted from each blood sample using a TRIzol-LS reagent. 12 cancer mRNAs biomarkers of interest were amplified, and the sequences were mapped to a human RNA reference sequence. They found that 20-42% of amplified sequences could be matched to human RNA for each biomarker, with between 5-10% of sequences that could not be matched. They found some of the biomarker targets that were identified from the extracted RNA were expressed at a concentration that was too low to have clinical value. These issues were reported to be due to the low quality of RNA that was extracted from the blood samples. Therefore, RNA extraction protocols have an effect on the

quality of RNA extracted which determines the ability to detect the target biomarker sequences.

A study in mice found that cancer mRNAs could be detected from exosome-like microvesicles in the blood (56). Mice were implanted with H460 human lung cancer cells to promote the growth of tumours. Tumour microvesicles were captured from the blood by antibody coated magnetic beads and the tumour specific mRNA sequences were reverse transcribed before being amplified by PCR. Using this technique, tumour specific mRNA sequences were detected in the blood of 40% of the tumour bearing mice.

PCR is a sensitive DNA and RNA sensing technique. However, it requires a complex, lengthy and sensitive sample preparation. To detect RNA expression levels adds an extra layer of complexity. The sample quality has to be pure RNA, which involves a complex RNA isolation procedure and then a lengthy processing of the RNA samples into cDNA. Both PCR and RT-PCR could result in sample loss, contamination or degradation, making this technique unpractical and unreliable for routine clinical use. Furthermore, PCR requires the isolation and lysis of cells of interest in order to do analysis and does not give analysis on the single cell level. This could result in the loss of information on rare cell sub-populations. Therefore, although PCR is a powerful technique at detecting specific DNA sequences, the complexity and length of the process limits its use as a routine method for clinical disease diagnostics (57).

There are currently clinical studies investigating the potential for liquid biopsy blood tests for cancer detection. Guardant Health is an American based company developing multiple different cancer screening methods for high risk patient populations for the early detection of cancer from liquid biopsies (58). They developed the Liquid biopsy Using NGS to Assay high-Risk patients (LUNAR) diagnostic test for colorectal cancer. LUNAR works through detecting genomic alternation and epigenetic cancer signatures through whole genome sequencing of circulating DNA. Guardant has generated disease signatures from data from over 80,000 patient samples collected from previous Guardant Health projects, allowing

the LUNAR project to focus on recurrent biomarkers. According to Guardant, LUNAR can detect genomic mutations at frequencies of 0.01% (59). In May 2019, Guardant Health announced plans to start a 10,000 patient trial to assess the ability to use the LUNAR test as a colorectal cancer screening technique. It has been reported that the market for Lunar in healthcare could be up to \$15 billion (60).

GRAIL is another company pushing forward for cancer diagnostics from a liquid biopsy. GRAIL utilises big data genomics, with vast amounts of tumour genome data obtained through whole genome sequencing (61). Instead of targeting gene expression changes, GRAIL detects changes in the DNA methylation, which is a method of controlling gene expression. Cancers can present an abnormal pattern of methylation, creating a biomarker to detect cancer specific signatures. GRAIL have created a large database of over 15,000 patients in the Circulating Cell-free Genome Atlas clinical study, that is currently on going (62). This study found that the sensitivity of GRAIL differs for different cancer types, with a sensitivity of 59% for lung cancer, 67% for ovarian cancer and 76% for oesophageal cancer and 86% for head and neck cancer (63). Furthermore, the sensitivity also depended on the disease stage, with sensitivities of 34%, 77%, 84% and 92% for stage 1, 2, 3 and 4 cancers, respectively (64).

LUNAR and GRAIL are two examples of research projects that are attempting to transition medical research into clinical practice. Therefore, there is an interest and a need for a technology that can detect cancers at early disease stages. The detection of ctRNAs and CTCs within liquid biopsies, such as blood, provides an attractive target.

1.4. Fluorescent mRNA Nanoprobes

Fluorescence is a property of some molecules to absorb light at a particular wavelength and emit light at a different wavelength, and this process can be described through a Jablonski diagram (Figure 4). When a molecule absorbs a photon, it is stimulated to an excited state at a higher vibrational energy level, where it exists for a finite amount of time. The excited molecule loses some energy

through non-radiative vibrational decay to the lowest vibrational energy level of the excited state, before decaying back to the ground state. This decay can happen in two ways: radiative decay is the release of energy in the form of a photon which results in fluorescence, while non-radiative decay occurs if this energy is lost through heat (65). When a photon is emitted from a molecule through radiative decay, this photon has a lower energy than the photon of light that was absorbed. This is called Stokes shift and can be visualised by the emitted photon displaying a shift to a longer wavelength, with lower energy, than the absorbed light. The fluorescence quantum yield is the probability that an absorbed photon will be emitted through radiative decay and generate fluorescence, rather than by non-radiative mechanisms and is the ratio between the number of photons emitted and the number of photons absorbed.

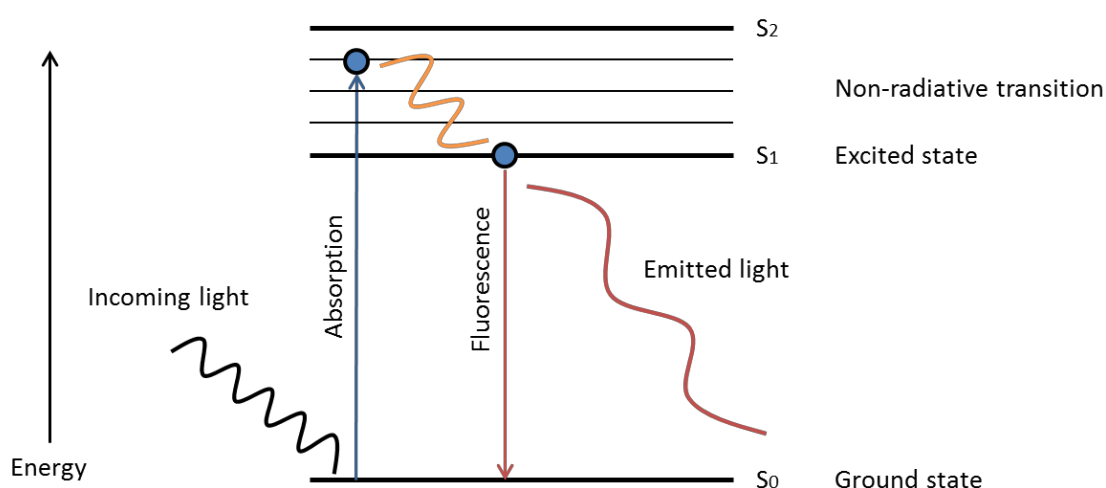


Figure 4. Jablonski diagram of fluorescence.

The Jablonski diagram illustrates the electronic states an electron goes through. A molecule is excited to a higher energy through the absorption of a photon of a specific wavelength. Some of this energy is lost through non-radiative decay. In fluorescence, the remaining energy is released through radiative decay in the form of a photon. The energy of the emitted photon is always less than the excited photon, therefore has a longer wavelength.

Fluorescent nanoparticles have become of great interest in molecular sensing and imaging. Molecular beacons, first reported in the late 1990's, have been developed as DNA sensors through the strong ability of DNA sequences to recognise and bind to a complementary target (66). These constructs function on a fluorophore-quencher pair within a hairpin DNA conformation and have a high specificity for hybridisation with target nucleic acid sequences (Figure 5) (67). Molecular beacons (MBs) have been discussed as promising fluorescence probes to detect nucleotide biomarkers. Reports have shown that molecular beacons are capable of imaging RNA in live cellular imaging, showing potential for nanoprobe in cellular uptake and biomarker detection (68).

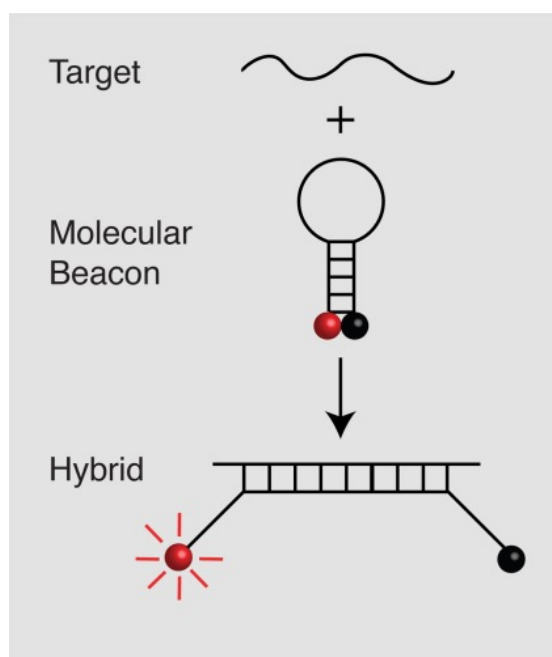


Figure 5. Molecular beacons for RNA sensing.

Molecular beacons are nucleotide sensing probes in a hairpin DNA structure. The stem of the molecular beacon brings a fluorophore (red) and quencher (black) in close proximity. The loop sequence acts as a target recognition and hybridisation with a target sequence results in a conformational change that increases the distance between the quencher and the fluorophore, which restores fluorescence. Figure used with permission, Copyright © 2012 Faculty of 1000 Ltd.

However, limitations of MBs include the method of delivery into cells. Efficient uptake is essential for the detection of target mRNAs and molecular beacons cannot be taken up effectively by cells without the use of a transfection agent, such as microinjection (69). This adds complexity to the sample preparation and can alter the cell membrane and result in cell death.

Nanoflares are a different type of fluorescent nanosensor that have been developed for nucleotide sensing. These utilise a different fluorophore-quenching pair from MBs, as the design is not based on hpDNA but consists of two oligo sequences, one of which is functionalised to a fluorophore and is initially quenched by the proximity to the gold nanoparticle. The recognition and binding to a target sequence results in the release of the fluorophore labelled oligo, which acts as a “flare” fluorescence signal (Figure 6) (70). Nano-flares are also capable of entering cells and act as an intracellular sensing device (71). Limitations of nanoflares include reports of non-specific fluorescence that results from the random release of DNA flare fragments which reduces the sensitivity of the probe and increases the background signal (72).

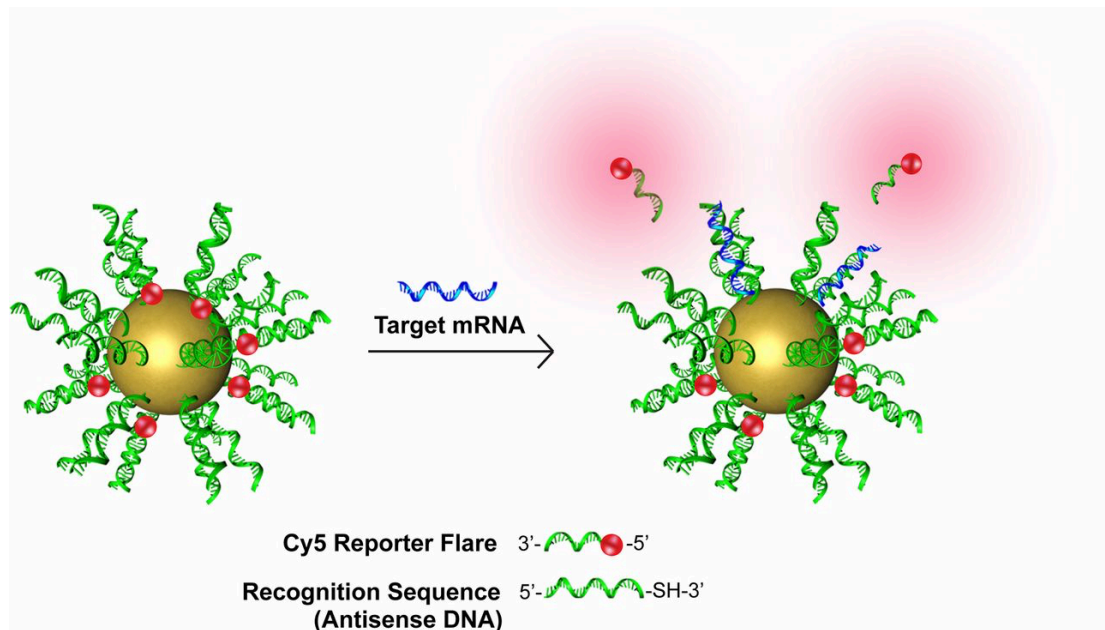


Figure 6. Nanoflares for mRNA detection

Nanoflares consist of spherical gold nanoparticles functionalised with target recognition DNA sequences. A fluorophore labelled (red) reporter flare sequence is hybridised to the recognition sequence and the fluorophore is quenched in close proximity to the gold nanoparticle surface. Upon the recognition sequence binding to the target sequence (blue), the flare sequence is displaced. This removes the fluorescence quenching and a fluorescence flare is released. Figure used with permissions, Copyright © 2014 National Academy of Sciences.

Table 1 displays the currently available methods of detecting mRNA biomarkers and the advantages and disadvantages of these methods.

Method	Advantage	Disadvantage
PCR	Highly sensitive Can detect low concentration of target	Complex and expensive methodology High risk of sample contamination
Cell Search	Easy use commercial kit	Low capture rates, misses
Size filtration	Rapid & easy methodology	Non-specific capture
Molecular beacons	Targeting loop provides high specificity target binding	Cannot enter cells, requires use of transfection agent
Nano flares	Can enter cells without transfection agent	High background signal Not capable of discriminating mRNA expression levels

Table 1. Advantages and disadvantages of current methods of mRNA biomarker detection.

In cancer research, fluorescent gold nanoparticles have become of interest in diagnostics due to their high specificity for target molecules and low toxicity. The applications of fluorescent labelled gold nanoparticles has shown promising results in tumour imaging, where targeted nanoparticles have displayed specific targeting and uptake in tumour tissue in mice (73). Furthermore, another technique that has been investigated are fluorescent nanoparticle “noses”, to detect protein cellular markers. This utilises nanoparticles coated in fluorescent polymers, which are

quenched when in close proximity to the gold surface, but competitive binding to the target protein releases the polymer and generates a fluorescent signal (74). Therefore, the application of fluorescent nanoparticles for molecular biomarker detection is a large and rapidly growing research area.

This project utilises fluorescence measurement techniques to characterise the properties of a GNR based fluorescence probe, hereinafter referred to as a nanoprobe. Fluorescence techniques are non-destructive, non-invasive and provide a rapid measurement output. Fluorescence also provides the ability for multiplex analysis, as different fluorophores of different wavelengths can be used to detect multiple biomarkers. The tuneable absorption properties of GNRs that creates their strong quenching abilities, combined with the advantages of fluorescence sensing techniques makes a fluorescence based GNR nanoprobe an attractive biomarker sensor.

1.5. Gold nanorod probes for fluorescence-based detection of mRNA biomarkers

The work in this thesis proposes a novel fluorescent nanoprobe for the detection of cancer mRNA biomarkers. The nanoprobe developed consists of a GNR core functionalised with fluorophore labelled, thiolated single stranded hairpin DNA (hpDNA). This hpDNA is formed through a complementary base paired stem which forms a targeting loop, which contains a complementary sequence to the target mRNA. In the absence of target mRNA, fluorescence from the fluorophore is quenched through energy transfer, due to the overlap between the fluorophore emission and the GNR absorption and the close proximity of the fluorophore to the GNR surface. In the presence of target mRNA, the DNA loop binds to the target mRNA which dissociates the stem binding. This results in the opening of the hpDNA, increasing the distance between the GNR and the fluorophore, which reduces fluorescence quenching and restores the fluorescence of the fluorophore (Figure 7). This design ensures that a fluorescence signal will only be generated when the nanoprobe comes into contact with its target.

The fluorescence quenching in the nanoprobe design relies on the spectral overlap between the longitudinal absorption of GNRs, which acts as the acceptor, and the emission of the donor, cy5. The fluorescence quenching in the hpDNA conformation is enhanced by the SPR of GNRs and following target mRNA hybridisation the fluorophore can undergo SPR enhanced fluorescence, providing an enhanced signal to background ratio.

This nanoprobe has a biological sensing element, the sequence of the targeting loop, and a signal transducer, the transition from quenched to unquenched state of the fluorophore. Benefits of using DNA as the sensing element are the endless variety of sequences combinations and the specificity of DNA base pairing. The target recognition sequence is composed of 15– 30 DNA bases, which forms the targeting loop, flanked by two short complementary stem sequences, which form the stem that holds the hpDNA structure. Fluorescence is a powerful signal transducer, as a range of fluorescence sensing mechanisms, such as steady state fluorescence spectroscopy, time resolved fluorescence spectroscopy, fluorescence microscopy and flow cytometry can be used to detect the signal. The fluorophore chosen in the nanoprobe design is cy5. Cyanine dyes display sharp absorption bands, high stability and resistance to photobleaching. They are commonly used to fluorescently modify DNA oligomers and it has been reported that the use of cyanine dyes at the 5' or 3' terminus of the sequences enhances the stability of the DNA structure (75).

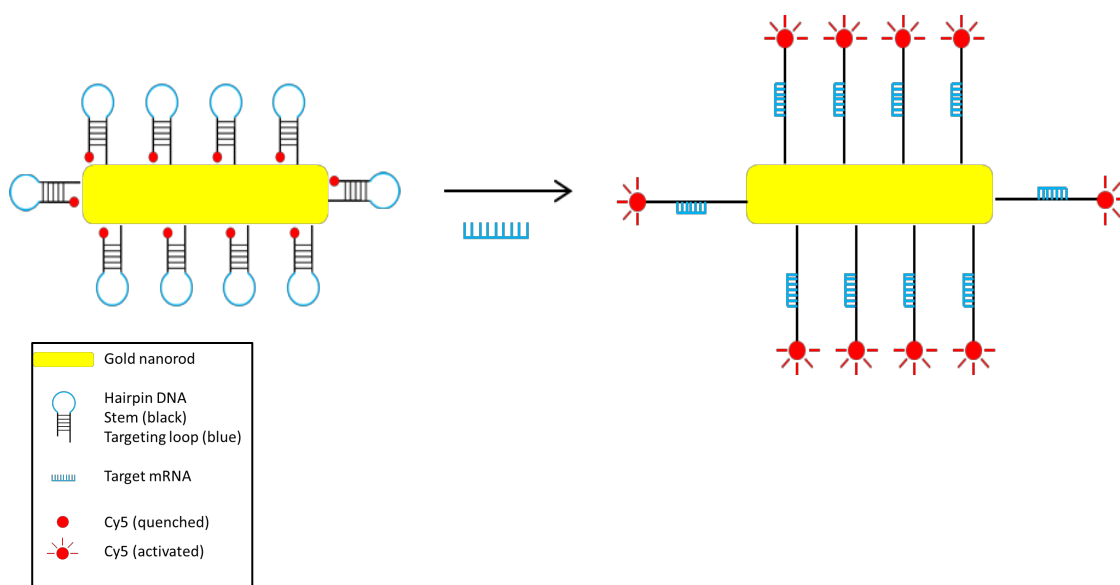


Figure 7. Schematic of the gold nanoprobe design.

Gold nanorods are functionalised with fluorophore labelled hpDNA. hpDNA utilises the stem-loop structure to position the fluorescence donor and the GNR acceptor in close proximity, resulting in FRET and fluorescence quenching in the closed hpDNA state. Upon targeting loop recognition of target mRNA, hybridisation occurs, dissociating the hpDNA structure. This increases the distance between the fluorophore and gold, reducing fluorescence quenching and restoring the fluorescence of the fluorophore.

The nanoprobe design depends on DNA hybridization, which offers a reliable, reproducible and controllable mechanism for target binding thanks to highly predictable base pairing and binding energy. The nanoprobe has initially been designed against C-MYC mRNA. Cancer is the sequential acquisition of mutations and dysfunctions of key cellular regulation genes. One of these key regulation genes is C-MYC, which normally functions to regulate cell division and cell survival. C-MYC has been reported to be overexpressed in over 40% of all human cancers (76) and this inappropriate gene activation promotes tumorigenesis through the uncontrolled replication and survival of cells. Therefore, C-MYC is an attractive molecular target for the detection of a variety of different cancer cell types.

As has previously been discussed, there are a range of techniques that have been developed for the detection of cancer biomarkers within blood, through CTC capture or ctRNA detection. However, these methods all have drawbacks that limits their clinical use. Many CTC capture methods rely on size filtration, which lacks specificity and results in the capture of non-specific cell debris. Commercially available kits have also been shown to have low capture rates, which results in the loss of data. Advances in PCR and DNA sequencing technologies have resulted in techniques to isolate and amplify DNA and RNA sequences from liquid biopsies. However, these techniques are still expensive and are susceptible to sample contamination and technical errors.

In comparison to these techniques, fluorescent gold nanoprobe incubated with a liquid biopsy to detect cancer biomarker mRNAs offers the potential for a high stability probe, as thiol labelled DNA can be bound to gold through a strong gold-sulphur bond. The nanoprobe offers the ability to detect a target with high specificity due to the complementary binding that occurs through DNA sequence recognition. Furthermore, the fluorescent signal generated from nanoprobe binding to target can be measured rapidly with simple and sensitive fluorescence measurement techniques.

The objectives of this thesis are to design and synthesise nanoprobe against mRNA cancer biomarkers of interest and investigate the potential of nanoprobe to detect target sequences by fluorescence measurement techniques. The end goal of this investigation is to develop nanoprobe for the detection of cancer biomarkers in liquid biopsies. The project initially investigated the nanoprobe binding to target in solution phase by fluorescence spectroscopy techniques, before progressing to investigations in cell lines and then patient liquid biopsies. The potential of nanoprobe to be used in liquid biopsies was investigated through flow cytometry fluorescence measurements. Flow cytometry provides the potential to perform fluorescence measurements on individual cells within large cellular populations, therefore the objectives of flow cytometry were to investigate the potential to

discriminate cancer cells from a background of non-cancer cells by the fluorescence generated by nanoprobe.

1.6. Conclusion

The results in this thesis will describe the synthesis and design of nanoprobe and their ability as a specific cancer biomarker sensor. Chapter 2 will introduce the experimental fluorescence techniques used in the project and the theory behind these methods. Chapter 3 will investigate the nanoprobe synthesis procedure and the optimisation of GNR ligand exchange and DNA functionalisation processes. The opening dynamics of the nanoprobe in solution phase was investigated in response to the addition of target DNA by steady state fluorescence and time resolved fluorescence spectroscopy and demonstrates the nanoprobe specificity to target mRNA. Chapter 4 investigates the cell uptake of nanoprobe and the applications of nanoprobe in detecting target mRNA in various cell lines using flow cytometry. Chapter 5 investigates the design of nanoprobe against different mRNA biomarkers of interest and the ability of nanoprobe to detect mutational biomarkers. Chapter 6 describes the fluorescence imaging of nanoprobe in cancer and non-cancer cells, which demonstrates nanoprobe uptake and further demonstrates nanoprobe specific fluorescence in target mRNA expressing cancer cells. Chapter 7 describes the preliminary results in testing the nanoprobe in cancer patient liquid biopsies, demonstrating proof of principle for nanoprobe detection of CTCs in a cancer diagnostic application. Finally, chapter 8 demonstrates the ability of nanoprobe to specifically detect target mRNA within exosomes extracted from a target mRNA cancer cell line, in comparison to exosomes extracted from a non-cancer cell line. These results demonstrate the potential for nanoprobe to detect cancer biomarker mRNAs in liquid biopsies that exist within both CTCs and ctRNAs within exosomes.

2. Experimental Methodology

2.1. Gold nanorod extinction spectra by UV-Vis spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy is a technique that measures extinction, which includes absorption and scattering properties. Extinction spectrum of GNRs were performed on a Perkin Elmer Lambda 2 spectrometer. A typical UV-Vis spectrometer contains a halogen lamp, which provides a light source for excitation between 300 – 1100 nm and a deuterium lamp for excitation from 190- 350 nm. The excitation light passes through a monochromator, which selects one wavelength of light to be exposed to the sample at a time. The light that is exposed to the sample can either pass through the sample or be absorbed by the sample. Figure 8 shows the typical setup of a UV-Vis spectrometer. The intensity of light that passes through the sample of interest (I) is compared to the intensity of light that passes through a reference sample (I_0). The absorbance at each wavelength ($A(\lambda)$) can then be calculated by the ratio between these intensities (Equation 2).

$$A(\lambda) = \log(I_0/I)$$

Equation 2.

An absorption spectrum from a GNR sample of interest can be used to calculate the GNR sample concentration. This uses the Beer-Lambert law (Equation 3), where ϵ is the molar extinction coefficient, b is the path length through the sample (cm) and c is the concentration (L mol). The molar extinction coefficient is a measurement of how strongly a chemical species attenuates light at a given wavelength. For GNRs samples, the extinction coefficient is a function of the longitudinal SPR and can be calculated from using the relationship obtained through the theoretical model (77). As nanorod aspect ratio increases, the longitudinal plasmon band increases in absorbance, therefore the extinction coefficient increases.

$$A(\lambda) = \epsilon \times b \times c$$

Equation 3.

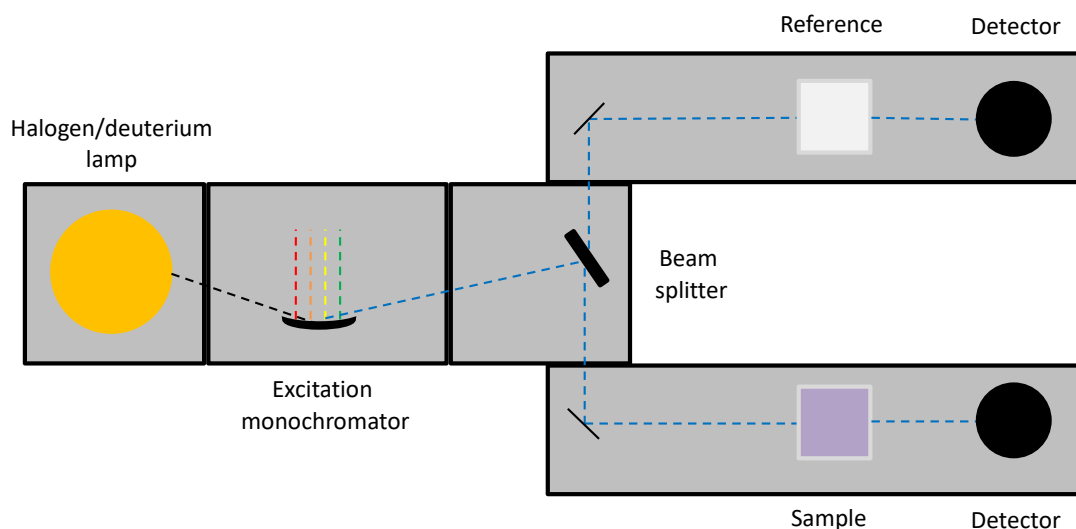


Figure 8. A typical set up of a UV-Vis spectrometer.

2.2. Steady state fluorescence spectroscopy

The fluorescence emission spectra of nanoprobe were measured on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon IBH Ltd., Glasgow, UK) (Figure 9). This utilises a broadband wavelength xenon light source which emits light that passes through an excitation monochromator to select the excitation wavelength of interest. The excitation light is exposed to the sample in the cuvette holder. The emitted fluorophores are collected at 90° from the excitation source, to avoid collecting excitation photons, and the emitted light passed through an emission monochromator to select the wavelength of light that is collected. The emitted light is then captured by a detector which converts the photon intensity into an electric signal.

Fluorescence spectroscopy was used as a quality assurance check after each nanoprobe synthesis. For each nanoprobe sample, the fluorescence emission intensity was measured five times before and after cDNA incubation and the average intensity was taken. Nanoprobes were only considered to be successful and used for further studies if there was a significant change in the fluorescence

emission following cDNA incubation, as compared with incubation with a random DNA sequence.

For accurate and reliable fluorescence emission measurements, it is important that both the lamp intensity and the monochromator alignment are regularly checked. For each measurement, the lamp should be warmed for 15 minutes prior to use to ensure stable output. Furthermore, the lamp intensity decreases with age and requires replacement after ~1000 hours of use, therefore a log is kept to track the lamp usage. The monochromators are calibrated with a reference sample which displays a peak at 467 nm. Based on this sample, the monochromator is set to 467 nm $\pm$ 0.5 nm to ensure the excitation light source is calibrated.

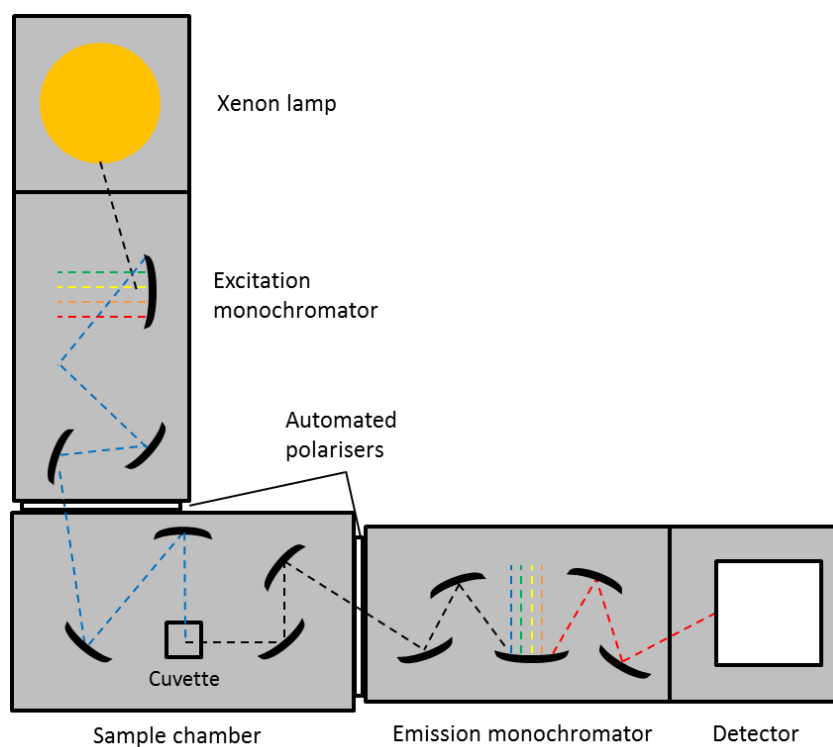


Figure 9. Schematic of steady state fluorescence spectroscopy.

2.3. Time resolved fluorescence spectroscopy

The fluorescence lifetime of a fluorophore is the average length of time it spends in the excited state before emitting a photon. This is represented as intensity versus time, which measures the amount of time between an excitation pulse and the return to ground state. The fluorescence lifetime of a fluorophore is independent of its concentration but is dependent on its molecular environment, including molecular interactions such as quenching.

Time resolved fluorescence lifetime measurements were obtained on an IBH Fluorocube fluorescence lifetime system (Horiba Jobin Yvon IBH Ltd., Glasgow, UK) (Figure 10). This consists of a pulsed excitation light source, a NanoLED, excitation and emission monochromators, automated polarisers and a detector. The excitation light passes through the excitation monochromator and a polariser at 0°. The excitation light is exposed to a fluorescent sample and the emission is collected at 90° to prevent the collection of excitation light. The light then passes through the emission monochromator, which is set to collect the emission wavelength of interest.

The fluorescence lifetime decay of a sample is measured by time correlated single photon counting (TCSPC). This utilises rapid laser pulses to excite a sample, where the laser excitation pulse is used as a “start” signal for the timing mechanism. The time between this excitation pulse being exposed to the sample and the emission of a photon is measured, with the emitted photon providing the “stop” signal. The time delay between excitation and emission is the fluorescence lifetime. This timing mechanism occurs on a picosecond and nanosecond time scale and creates a histogram decay profile based on the emitted photons. The histogram of the fluorescence decay is created through the time to amplitude converter (TAC). This process is repeated many times and the measurement stops when one channel has a peak count of 10,000 photons.

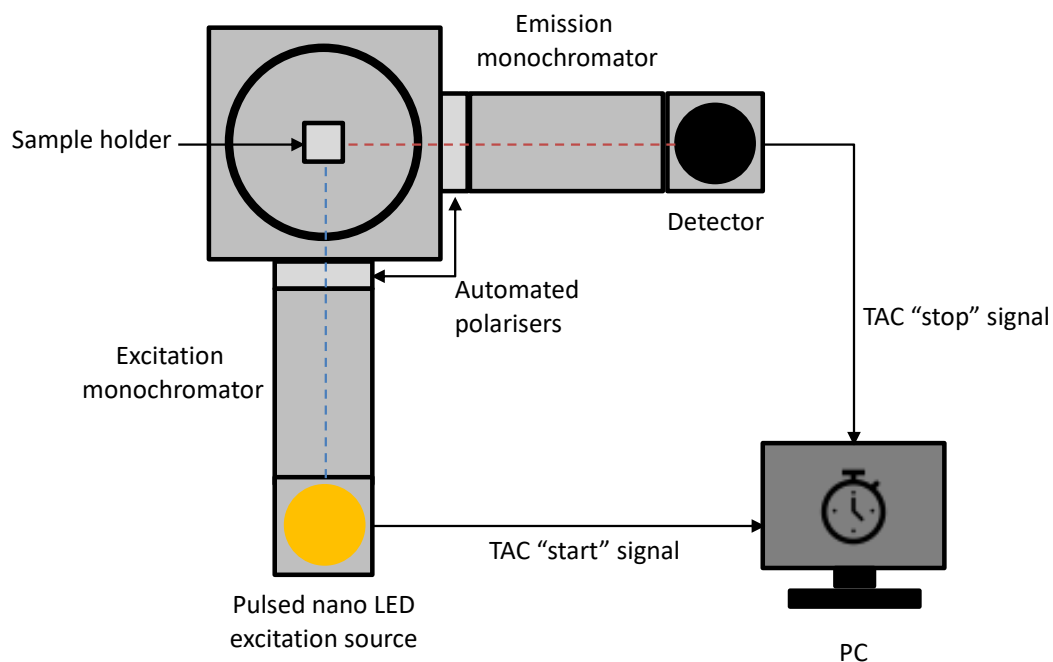


Figure 10. Schematic of TCSPC by time resolved fluorescence spectroscopy.

If more than one fluorophore exists in the sample, then the fluorescence decay is a sum of the different lifetime species. To extract the fluorescence lifetimes within the sample, the histogram is fitted with exponential decay functions. This process extracts the fluorescence lifetimes present and the relative contribution of each species within the sample. The photon time distribution histogram was fitted with a multi-exponential decay model. Each fluorescence lifetime is extracted by a decay function (Equation 4). In this model A represents the pre-exponential weighting factor, B is fluorescence lifetime amplitude, t is time and τ represents the exponential decay lifetime value. The fluorescence decay of a sample is the sum of the exponential components (Equation 5).

$$F(t) = A + B_1 \exp\left(\frac{-t}{\tau_1}\right)$$

Equation 4.

$$I(t) = \sum_i^n B_i \exp\left(\frac{-t}{\tau_i}\right)$$

Equation 5.

The “goodness of fit” of the decay function is calculated between the measured fluorescence decay and the fitted decay curve. This is calculated using the non-linear least squares method. The goodness of fit is given by the χ^2 value, with a value of 1 indicating a perfect fit. However, values ranging from 0.9 to 1.2 considered to be a good fit. Values greater than 1.2 would indicate that an inappropriate model has been used to fit the decay curve. This calculation for χ^2 is shown in Equation 6, where $N(t_k)$ is the measured data, $N_c(t_k)$ is the calculated decay data and σ_k is the standard deviation of each data point.

$$\chi^2 = \sum_{k=1}^n \frac{1}{\sigma_k^2} [N(t_k) - N_c(t_k)]^2$$

Equation 6.

The goodness of fit is also demonstrated by the residuals of the decay fitting. The residuals display the distribution of the data around the fitting and these should be low in count and display a random distribution. The error of a lifetime decay fit is calculated by multiplying the standard deviation of the decay fit by three, which provides a high confidence interval that the decay fit represents the lifetime distribution within the sample.

2.4. Flow cytometry

Flow cytometry is a powerful fluorescence technique that allows measurements to be carried out on individual cells in a liquid stream. It is a non-invasive procedure that uses lasers to analyse the physical and chemical characteristics of individual

cells. Figure 11 demonstrates the principle of flow cytometry measurements. Cells move in single file through a high-pressured stream and pass across a laser beam. As the cell passes through the laser, light is scattered in many directions. Forward scatter (FSC) is dependent on the size of the cell as large objects will refract more light, therefore generate a higher FSC signal. Side scatter (SSC) is dependent on elements within the cell such as granules or nanoparticles that can also scatter light.

As well as cellular scattering properties, the fluorescence of a cell can also be detected. If cells contain a fluorophore that is excited by the laser beam, the emitted light is passed through filters separate the light emitted by the fluorophore before a photomultiplier tube converts this into an electrical intensity signal. The fluorescence released from the cell is measured as a cumulative signal; therefore, the total fluorescence from a cell can be measured but not the localisation of the signal. This signal is then presented on a histogram, where the intensity of the signal is on the x-axis with the number of cells displaying that signal on the y-axis.

Nanoprobe uptake and fluorescence was measured in cells by flow cytometry, using a Beckman-Coulter MoFlo XDP cytometer. This cytometer is equipped with 3 lasers of 336 nm, 488 nm and 640 nm. Figure 12 shows the design and detection pathways of this flow cytometer. Forward scatter is measured straight on from the sample stream with a 488 nm laser. Side scatter and fluorescence signals are measured at a 90° angle from excitation. Side scatter is measured with a 488 nm excitation with a 505 nm short pass filter and a 488/10 bandpass filter. Nanoprobe fluorescence from cy5 was measured with 640 nm excitation with a 700 nm short pass filter and a 670/30 bandpass filter (FL8) (78).

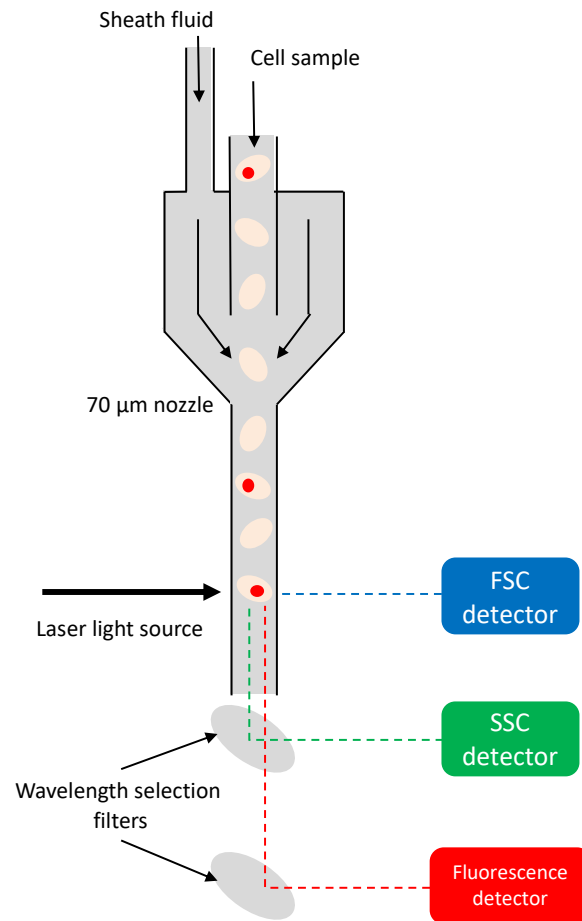


Figure 11. Principles of flow cytometry.

The scattering and fluorescence properties of a cell can be detected by flow cytometry. A fluorophore within a cell (red dot) is excited by a laser and the emitted photons pass through optical filters to be collected by a detector.

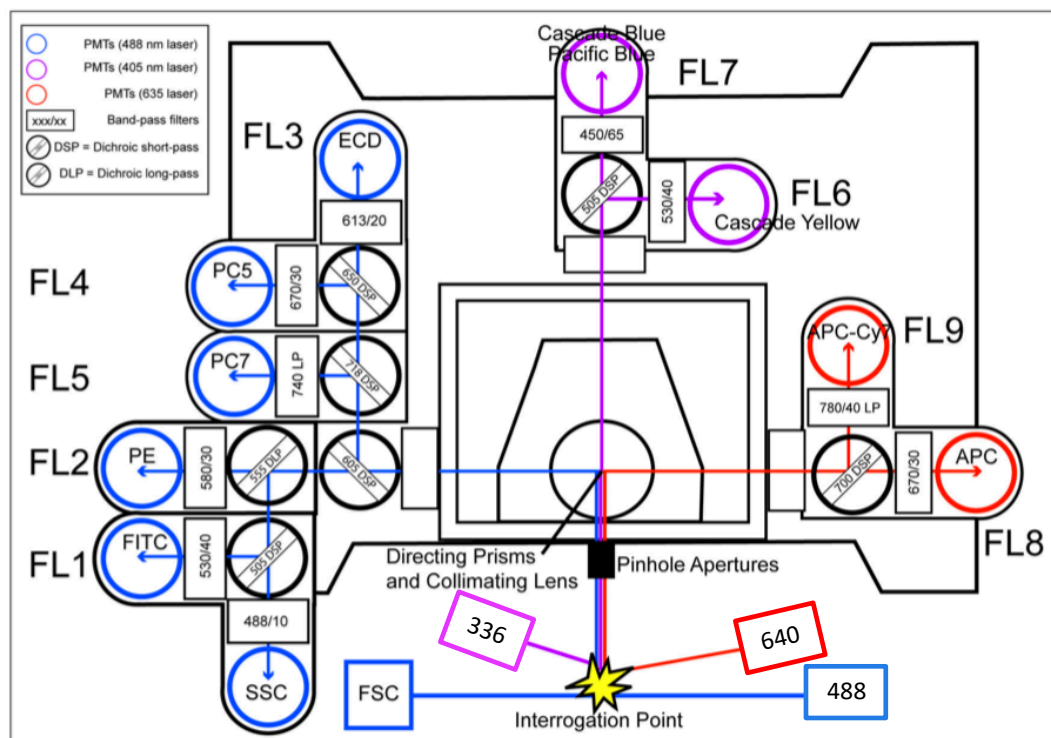


Figure 12. Schematic of the MoFlo XDP detection pathways.

After cells are exposed to the laser light, the scattered and emitted light goes through wavelength selection filters, to be received by the appropriate detector. The forward scattered light is collected straight ahead of sample exposure, while side scattered light and fluorescence emission are collected at 90° from sample exposure. This figure was adapted from the Beckman Coulter MoFlo XDP instruction manual.

Laser alignment was performed with Flow-check pro fluorospheres, which consists of a mixture of different sized fluorescence beads with different emission profiles. This mixture contains 10 µm fluorospheres with a fluorescence emission of 515 to 800 nm when excited at 488 nm, 6 µm fluorospheres with a fluorescence emission of 640 to 800 nm when excited at 635 nm, and 3 µm fluorospheres with a fluorescence emission of 400 to 500 nm when excited at 405 nm (79). This allows the calibration of the cytometer, as the beads are used to adjust the laser position

and intensity until clearly defined populations are observed, based on their size and fluorescence.

It is important to consider that flow cytometry measurements are not absolute values but are relative, and the measurement output can be increased or decreased according to the voltage applied. The emitted fluorophores from individual cells is detected by a photomultiplier, which converts photons into an electrical signal, and this is recorded as fluorescence intensity. The electrical signal strength is dependent on the number of photons detected. The signal intensity can be amplified by applying a voltage to the photomultiplier, enhancing the electrical current (80). Therefore, it is important to carry out laser alignment before each measurement to ensure the lasers are hitting the sample stream and it is essential to keep the voltage settings consistent between sample measurements in order to make a direct comparison. Generally, a voltage is chosen which sets the control cells to a low scale to make the signal: background ratio high. Increasing the ratio beyond this point will result in increasing the background signal.

2.5. Confocal fluorescence imaging

Fluorescence imaging was performed on a confocal microscope (Leica Microsystems, SP8 + picoEMERALD S + CW vis lasers) using LASX software. In confocal microscopy, laser light is focused onto a defined spot at a specific depth within a sample, which is the focal point. A pinhole is used to cut off signals that are out of focus, allowing only emitted light from the focal point to enter the detector. When the emitted light reaches the detector, this transforms the light signal intensity into an electrical signal. By scanning across an area of interest this electrical signal is used to build up the image intensity, pixel by pixel. Figure 13 depicts the typical set up of a confocal microscope for fluorescence imaging within a cell.

By scanning across a sample in the x and y directions, an image of one optical plane is generated across a sample. This enables sharp imaging of a focal plane within a cell, without fluorescence background from other regions within the cell. Confocal

microscopy allows the imaging of cellular structures with high resolution. It also allows the analysis of 3D images, by stacking several images from different z positions within the optical plane.

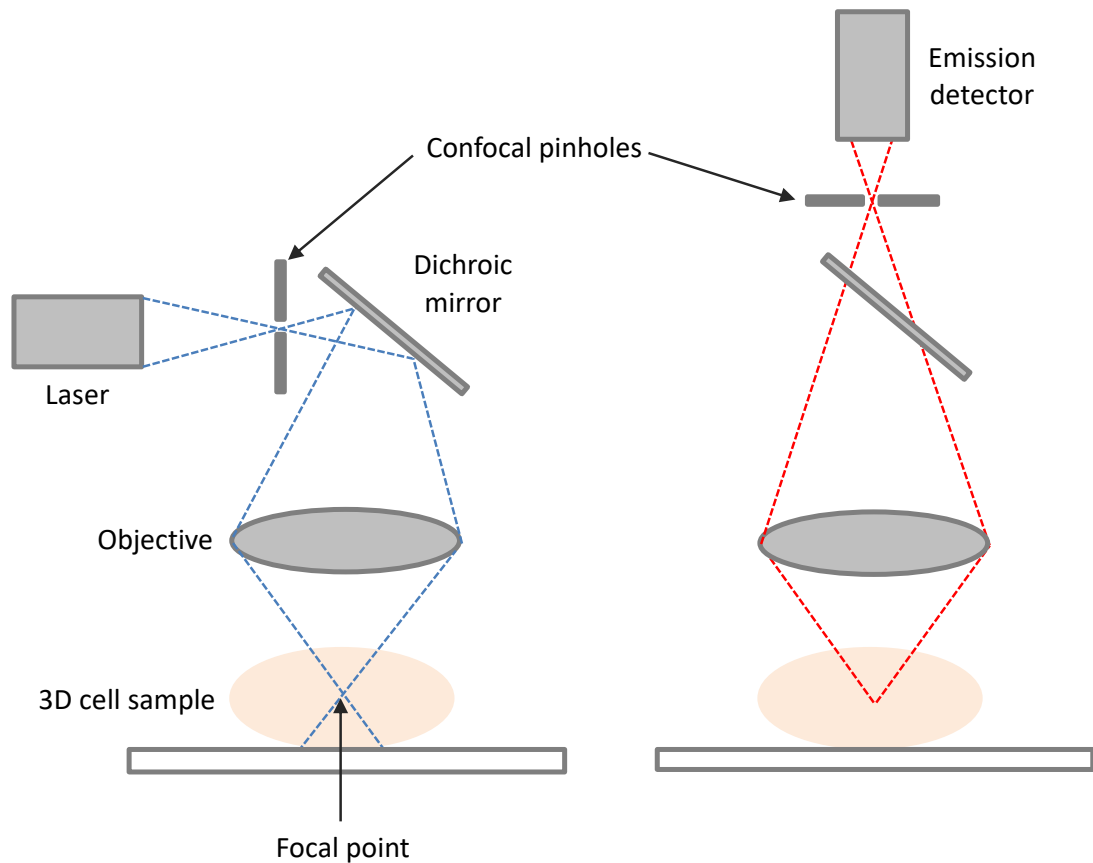


Figure 13. Fluorescence imaging using a confocal microscope.

The laser excitation pathway is focused onto a specific point within a defined focal plane (blue). The emission photons from the focal point are collected by the emission detector (red). This process is repeated across an area of interest, to build up a fluorescence intensity image pixel by pixel.

2.6. Fluorescence lifetime imaging microscopy

As well as measuring the fluorescence lifetimes within a solution, TCSPC can also be used to determine the fluorescence lifetimes within an image by fluorescence lifetime imaging microscopy (FLIM). In FLIM, TCSPC measures the time between

sample excitation by a pulsed laser and the arrival of the emitted photon at the detector. TCSPC requires a defined “start”, provided by the electronics steering the laser pulse or a photodiode, and a defined “stop” signal, realized by detection with single-photon sensitive detectors. The measurement of this time delay is repeated many times to account for the statistical nature of the fluorophore’s emission. The delay times are sorted into a histogram that plots the fluorophore emission over time after the excitation pulse. Using the same equation as fluorescence spectroscopy (Equation 5), the fluorescence lifetimes within each pixel can be extracted by using a single or multi-exponential decay model.

In order to acquire a fluorescence lifetime image, the photons have to be attributed to the different pixels, which is done by storing the absolute arrival times of the photons additionally to the relative arrival time in respect to the laser pulse. Line and frame marker signals from the scanner of the confocal microscope are additionally recorded in order to sort the time stream of photons into the different pixels.

Figure 14 demonstrates a typical FLIM setup within a confocal microscope. This consists of a pulsed laser source, a dichroic mirror to separate the excitation and emission wavelengths, an objective to focus the excitation light onto the sample, a single photon sensitive detector and a TCSPC unit to measure the time between excitation and fluorescence emission.

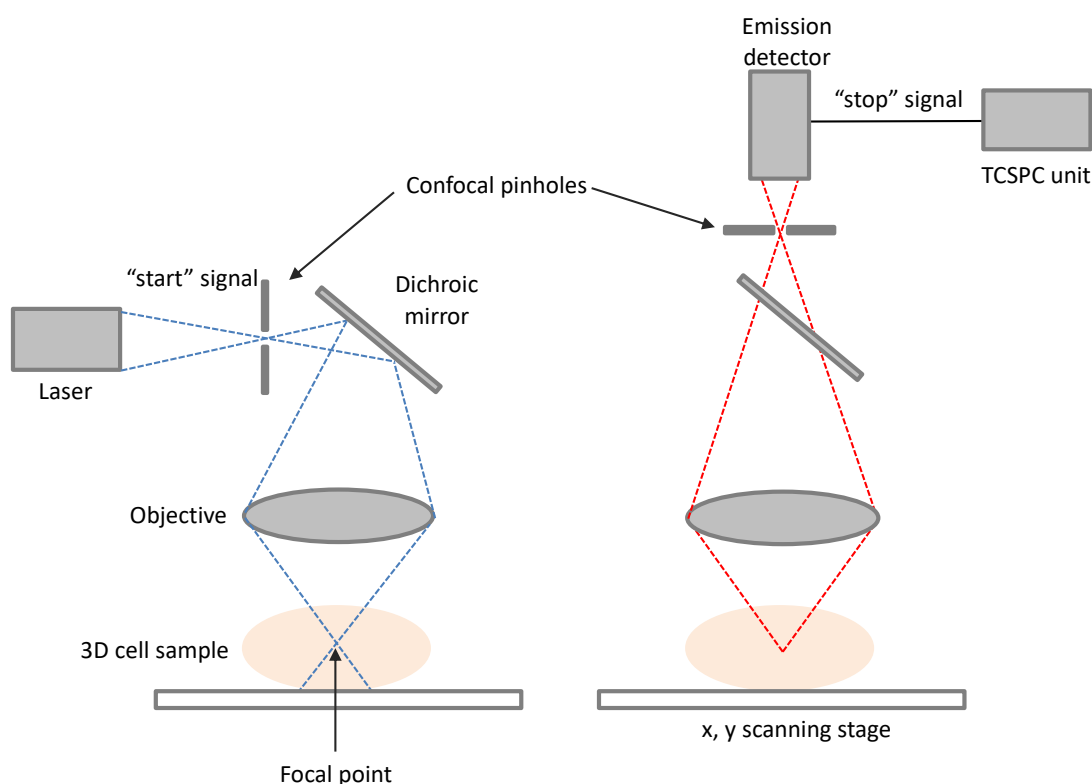


Figure 14. A typical configuration of fluorescence lifetime imaging microscopy.

The laser excitation pathway through a confocal microscope excited a specific point in a defined focal plane within a cell (blue). The emitted photons from the focal point are collected by the emission detector (red). The time from laser excitation to the collection of an emitted photon is recorded through the TCSPC unit. This process occurs repeatedly by scanning across a region of interest, to generate fluorescence lifetime decay profiles in individual pixels.

3. Optimising gold nanoprobe synthesis and investigating nanoprobe opening dynamics by fluorescence spectroscopy

3.1. Introduction

The optical properties of GNRs are tuneable from visible to near-infrared region of light (600- 1000 nm) and this is controlled by the nanoparticle shape and size (81). Nanorods exhibit two plasmon bands; the longitudinal band which corresponds to light absorption and scattering along the long axis, and the transverse band, corresponding to the light absorption and scattering along the short axis of the nanorod. It is the longitudinal band that can be tuned to a wavelength of choice during the synthesis procedure.

In order to control nanorod synthesis, an understanding of the synthesis chemistry is required. It has taken over a decade to develop and fully understand the synthesis of GNRs. This seed-mediated growth method is the most established synthesis procedure, which was first described in 2001 (82).

The synthesis of GNRs is now relatively well understood and involves a multistep redox reaction. Firstly, small gold seed particles of ~4nm are produced from a gold salt solution, HAuCl_4 , which contains Au^{3+} , in the presence of a surface stabilising surfactant cetyltrimethylammonium bromide (CTAB). The gold ions are reduced by sodium borohydride into Au^0 . This seed solution is then added to a larger growth solution, which contains all of the reagents required for rod formation. The growth solution contains more gold salt in a solution of CTAB, ascorbic acid and silver nitrate (83).

CTAB is a surfactant and acts as a surface stabilizer to promote anisotropic growth of the nanorods (84). The surface of a gold crystal contains 5 side faces (110) and 10 end faces (111). The side faces (110) contain higher surface energy which promotes the CTAB hydrophilic groups to bind preferentially to these areas. This promotes the growth of rod-shaped gold nanoparticles, as CTAB binding to the side faces constricts seed mediated growth to the end (111) faces. CTAB has less affinity

to bind to the end (111) faces, therefore the coating of CTAB on the tips of GNRs is thinner. The hydrophobic tail of the exposed CTAB is bound by another CTAB molecule, creating a bilayer on the surface of the GNR (85). GNRs coated by a bilayer of CTAB are very stable as the positively charged surface coating prevents nanoparticle aggregation due to electrostatic repulsion.

Ascorbic acid is a reducing agent that is added to the growth solution, resulting in the reduction of gold salt into gold ions required for growth. However, ascorbic acid is a weak reducing agent and it has been reported that only ~15% of the total gold in the growth solution is incorporated into GNRs (86). Therefore, a large amount of growth solution is required to produce a moderate concentration of GNRs (87). In the presence of CTAB, gold seed particles are grown into rod shaped particles with a low yield. When silver nitrate is added to the growth solution, rod shaped particles are formed at a high yield, as compared to spherical particles. Until recently, the role of silver nitrate in the GNR synthesis procedure was not fully understood. However, it was known that the addition of silver results in an improved GNR yield and shape monodispersity, producing over 90% rod shaped particles (88). Furthermore, increasing the silver nitrate concentration in the growth solution results in GNRs with increased aspect ratios (89). Recently, electron microscopy studies of GNR growth have uncovered the mechanism by which silver nitrate effects the final aspect ratio of GNRs. It was found that when the small gold seed particles reach a critical mass of 4 to 6 nm in diameter, silver nitrate deposition is more favourable on the side (110) facets which promotes rod-shaped growth. It was also found that increasing the silver nitrate concentration resulted in rod formation with smaller gold seed particle diameters and this is the mechanism that influences the final aspect ratio, as the average width of the GNRs increases as the final amount of silver nitrate decreases (90).

Cellular uptake is an important factor to be considered in the nanoprobe design, as the nanoprobe has to be intracellular to come into contact with target mRNA. Many studies have investigated the effect of shape, size and surface chemistry on the biocompatibility and uptake of GNRs into cells (91–93). GNRs have been previously

been reported to be more cytotoxic than nanospheres (94), however it is now believed that this cytotoxicity arises from excess CTAB from the synthesis reaction, rather than the GNRs themselves (95,96). There have been many reports of the cytotoxicity that is induced by GNRs coated in CTAB (GNR-CTAB), with cell lines displaying almost 100% cell death after 24-hour exposure (97). Furthermore, the tightly packed CTAB bilayer also hinders functionalisation of the GNR surface for biological applications. Studies have investigated modifying the GNR surface to exchange CTAB for biocompatible molecules to reduce the toxic effects. This has shown that nanoparticles functionalised with cationic surface charges were moderately toxic to cells, whereas gold nanoparticles functionalised with anionic surface coatings were nontoxic (98). Therefore, the composition and charge of surface ligands has an effect on the nanoparticles uptake and toxicity.

Following GNR synthesis, a ligand exchange process was carried out in order to remove the CTAB bilayer and to make the GNRs biocompatible. The CTAB bilayer was removed and GNR were coated with mercaptohexanoic acid (MHA), through a round-trip phase transfer ligand exchange process (99). This procedure firstly extracts GNRs from an aqueous to organic phase, where CTAB is displaced by dodecanethiol (DDT). GNRs-DDT were then extracted back into the aqueous phase, where DDT is exchanged for MHA. MHA is a short chain thiolated carboxylic acid that binds to the gold surface through the thiol group, resulting in a strong and stable gold–sulphur bond (100). MHA coated GNRs (GNR-MHA) are stable, biocompatible (101) and can be functionalized for nanoprobe applications.

DNA functionalisation of GNRs has received a lot of interest for the potential use in disease diagnostics and treatments. DNA nucleotides make an ideal framework for molecular probes. They are simple to synthesize, the sequence can be designed and modified, and they have a high binding affinity and specificity. A stable conjugation between the DNA and gold is essential for the probe design and mRNA sensing abilities. The DNA used in the probe design was designed with a thiol group modification, which facilitates binding to the GNR surface through a gold-sulphur

covalent bond (102). DNA functionalisation using thiol as an anchor group has been demonstrated to have the highest thermal stability than using other sulphur containing groups, such as acyclic disulphide and cyclic disulphide (103).

There is a vast range of protocols for the DNA functionalisation of nanoparticles, with the majority utilising a “salt-aging” process. MHA-GNRs exhibit a negative surface charge, as do DNA molecules. Therefore, they repel each other and cannot naturally establish bonds. Sodium ions (Na^+) are required to facilitate this attachment, which occurs when GNRs are present with excess amounts of DNA and the gradual addition of Na^+ ions. This is a highly sensitive process; as if the concentration of Na^+ becomes too high the ions will bind directly to the surface of the GNRs, which results in the irreversible aggregation of GNR particles. It has been described that the salt-aging process is more challenging for large GNRs of over 50 nm, which require a longer salt aging process and are more sensitive to aggregation, compared with smaller nanoparticles (104). The end goal from the gradual salt-aging process is to produce GNRs with a high density of attached hpDNAs. The process from GNR synthesis from gold seed particles, to ligand exchange to DNA functionalisation is represented in Figure 15.

However, there does not seem to be an agreed on or standardised method of salt-aging. The original salt-aging protocol was described in 1996, and involved a lengthy salting process, over several days and required a careful control of ionic concentration (105). Since then, advancements have been made in understanding the DNA attachment process and modifications to the protocol have been made. The introduction of surfactants, such as SDS, has increased the speed of salt aging as they reduce the aggregation of nanoparticles, making it possible to reach high salt concentrations and increasing reproducibility. Another protocol describes the production of DNA functionalised gold nanoparticles by incubating the gold nanoparticle-DNA mixture in 0.1 M of NaCl and increasing the NaCl concentration every 4 hours by 0.1 M increments (106). This allows a faster DNA functionalisation at higher salt concentrations.

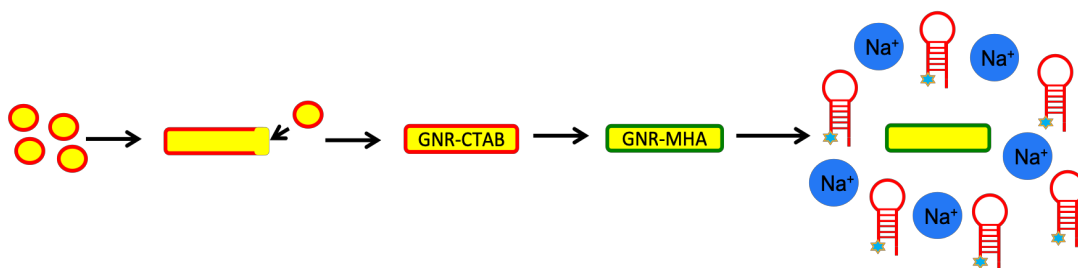


Figure 15. Process of gold nanorod synthesis, ligand exchange and DNA functionalisation.

In order to make a nanoprobe, gold nanorods are first synthesized by the seed-mediated silver assisted growth process. This produces GNRs coated in a bilayer of CTAB. In order to make GNRs biocompatible, a ligand exchange process is performed which removes the CTAB layer and replaces this with MHA. These GNRs can then be functionalized with fluorophore labelled hpDNA by a salt-aging process, in which sodium ions are gradually added to promote DNA attachment to GNRs.

Hurst, et al studied the effect of salting buffer parameters on DNA loading (107). They found that DNA loading was independent of the speed of salting but was dependent on the conditions of salting. Nanoparticle samples that underwent a brief sonication of 10 seconds after each salting buffer addition were found to have an enhanced DNA attachment. It is thought that sonication improves DNA attachment by creating movement and space within the solution, which can promote interactions between the gold and thiol, increasing DNA attachment. They also confirmed that DNA strands were not degraded following brief sonications.

New conjugation methods have been reported that speed up the DNA attachment process. A rapid DNA attachment process was reported by Zhao et al., in which gold nanoparticles were stabilised by a layer of mononucleotides. A temperature facilitated ligand exchange process was then facilitated to exchange the mononucleotide layer with thiol-DNA strands, in a high salt environment. Using this approach, DNA functionalisation was achieved within 4 hours (108).

In 2012, the first nanoparticle DNA functionalisation paper discussed the effect of pH on DNA attachment (104). They demonstrated that the pH of the DNA attachment reaction mixture had a profound effect on the sample stability. When citrate capped nanoparticles were incubated with DNA and NaCl, the sample at pH 7.6 displayed rapid aggregation, whereas the sample at pH 3 remained stable. 80% of DNA adsorption was found at pH 3, compared to 40% at pH 7.

However, these publications all focus on gold nanospheres, and very little literature exists on the optimal conditions for DNA attachment to GNRs. In 2013, a report supported the idea that a low pH was important for DNA functionalisation and demonstrated this with GNRs (109). A rapid DNA functionalisation protocol was developed for CTAB coated GNRs, when the salting buffer was prepared at pH 3. This offers promising results that DNA functionalisation of GNRs can be improved by altering the pH of the salt aging process.

Once the nanoprobe is synthesised, characterisation measurements need to be performed to determine the sensitivity and specificity of target binding. Fluorescence spectroscopy is a powerful technique for the detection of biomolecules, offering rapid and sensitive detection. The use of fluorescence is dependent on the physical properties of the detected fluorophore, such as its photostability, emission maximum and fluorescence lifetime. The use of FRET in the nanoprobe design provides an “off-on” fluorescence signal, which offers real-time and sensitive information on the dynamics of the nanoprobe. This FRET design has already been demonstrated to work using gold nanospheres, with the probe displaying initial low fluorescence intensity, which increases after the addition of complementary DNA (cDNA) (48).

The objective of this study was to synthesise GNRs, exchange their surface coating from CTAB to a biocompatible ligand and functionalise GNRs with fluorophore labelled hpDNA. The ability of these nanoprobe to detect target sequences was then investigated through fluorescence spectroscopy in solution phase. Although GNR synthesis and ligand exchange protocols are well known, there is a knowledge

gap in the DNA functionalisation process for hpDNA to GNRs. This chapter will validate GNR synthesis and ligand exchange protocols to ensure they are reliable to produce stable GNRs. The DNA functionalisation process was then investigated, to optimise the conditions for maximum DNA attachment to GNRs. The pH value of the DNA functionalisation buffer was found to be critical for the attachment of DNA, with nanoprobe produced at a lower pH value demonstrating a higher ability to detect target DNA. The specificity of the nanoprobe to a target sequence was demonstrated through steady state and time resolved fluorescence spectroscopy, with a high fluorescence response from the nanoprobe following target DNA incubation but this was not observed following nanoprobe incubated alone or incubated with random DNA.

3.2. Sample preparation and characterisation

3.2.1. Materials

Hexadecyltrimethylammonium bromide (CTAB), gold(III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) (520918), sodium borohydride (NaBH_4), silver nitrate (AgNO_3) (209139), L-ascorbic acid (A1968), dodecanethiol (DDT) (471364), acetone (650501), toluene (244511), methanol (32213), mercaptohexanoic acid (MHA) (674974), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (C4706), sodium dodecyl sulfate (SDS) (436143), sodium phosphate monobasic (NaH_2PO_4) (S5011), sodium phosphate dibasic (Na_2HPO_4) (S5136), sodium chloride (59888), ethanol (32221), isopropanol (I9516) were purchased from Sigma-Aldrich. Thiolated oligonucleotides and the corresponding complementary oligonucleotides were purchased from Sigma custom DNA oligos.

3.2.2. Gold nanorod synthesis

Gold nanorods were synthesised following the silver-assisted seed mediated growth procedure. To produce gold seeds, 7.5 ml of 0.2M CTAB was mixed with 2.5 ml of 0.001 M HAuCl_4 for 3 minutes before 0.6 ml of 0.01 M NaBH_4 was added and vigorously mixed with a magnetic stirred for 3 minutes, to form an orange-brown

seed solution, which was matured for 3 hours at room temperature. The growth solution was then prepared by mixing 200 ml 0.2 M CTAB, 200 ml 0.001 M HAuCl₄, 8.8 ml of 0.004 M AgNO₃ and 2.8 ml of 0.0778 M ascorbic acid. The addition of ascorbic acid results in a change from an orange to colourless solution. 0.4 ml of seed solution was injected into the growth solution and left at room temperature, in the dark, overnight to produce GNRs coated in CTAB (GNR-CTAB). The GNR solution was then centrifuged at 12,000 xg for 30 minutes twice and re-suspended in distilled water to remove excess CTAB.

3.2.3. Gold nanorod ligand exchange

Ligand exchange was performed following the round-trip phase transfer. 1ml of 40 nM GNR-CTAB were mixed with 1 ml of DDT and 4 ml acetone, to extract the GNRs from aqueous to organic phase. GNR coated in DDT formed a layer, which were extracted. Excess DDT was then removed by adding 1 volume of toluene and 5 volumes of methanol. The sample was mixed by swirling and left for 1 hour for the GNRs to precipitate. The precipitated GNR-DDT were extracted by removing the clear methanol-toluene liquid and resuspended in 1 ml of toluene. GNRs were extracted back to the aqueous phase by heating at 90-95°C with 0.01 M MHA in 9 ml of pre-warmed toluene, with vigorous stirring in a water bath with a magnetic stirrer until aggregates were visible, which occurred within 10 minutes. GNR-MHA were washed twice in toluene, by centrifuging for 5 minutes at 8000 rpm, and finally resuspended in TBE buffer.

3.2.4. Gold nanorod DNA functionalisation

Hairpin DNA (hpDNA) is a single stranded DNA sequence that is comprised of a middle DNA targeting loop and two complimentary ends that form into a stem. hpDNA containing a 5' cy5 dye was designed against the target gene mRNA sequence and custom oligos were ordered from Sigma Aldrich. Complementary DNA (cDNA) sequences were also designed, which were the reverse complement DNA sequence of the targeting loop of the hpDNA.

Firstly, the disulphide bonds of the hairpin were reduced by incubating DNA with 10 mM TCEP made up in 1x TE buffer with a molecular ratio of TCEP:DNA at 100:1 for 1 hour at room temperature with shaking on a sample shaker at 800 rpm. The DNA was then precipitated in 0.3M NaMg acetate and 70% ethanol by incubating at -20° for 3 hours. DNA was centrifuged at 13,000 rpm for 15 minutes at 4°C and the supernatant was removed before the DNA fluorescent pellet was resuspended in 100 µl of distilled water. GNRs were conjugated with DNA through a salt aging process. From previous studies, the optimum ratio of GNR:DNA used was 400:1 (110). 5 nM of GNRs were mixed with hpDNA, 0.02% SDS and 10 mM Na phosphate pH5 overnight with shaking. For every 200 µl of conjugation solution, 50 µl of salting buffer was added, at intervals of 10 µl an hour. Salting buffer was composed of 0.5 M NaCl, 0.02% SDS and 10 mM Na phosphate pH5. After 100% salting was achieved the mixture was incubated overnight with shaking. GNR-hpDNA was purified by centrifugation at 13,000 rpm for 15 minutes at 4°C. The conjugate was then washed three times with washing buffer (10 mM Na phosphate buffer pH7.5 + 0.02% SDS) followed by three washes with 10 mM phosphate buffer pH7.5. Each wash consisted of a 15 minute centrifugation at 13,000 rpm at 4°C, followed by supernatant removal and resuspension of the DNA pellet. The GNR-DNA probe was finally re-suspended in 10 mM phosphate buffer pH7.5 and the concentration of nanoprobe was determined by UV-Vis spectroscopy.

3.2.5. Steady state fluorescence spectroscopy

The fluorescence emission spectrum was measured using a Fluorolog-3 spectrofluorometer with excitation at 638 nm and a 5 nm slit. The measurement range was set from 650 nm to 750 nm to collect the emission from cy5. Each sample measurement was repeated 5 times and averaged to obtain an emission intensity graph. Measurements were taken with nanoprobe alone and then 2 hours after incubation with cDNA at 1000:1 ratio.

3.2.6. Time resolved fluorescence spectroscopy

The fluorescent lifetime of the probe was measured by time correlated single photon counting on an IBH Fluorocube fluorescence lifetime system (Horiba Jobin Yvon IBH Ltd., Glasgow, UK). A pulsed light emitting diode of 638 nm was used as the excitation source at 1 MHz. MHA-GNRs at OD= 0.2 were measured as a prompt and the emission monochromator was used to collect emission at 638 nm and the emission polariser was set to 0°. An emission monochromator was used to select the emission wavelength and the nanoprobe decay curves were recorded at emission 665 nm and the emission polariser was set to the magic angle of 54.7°. Excess complementary DNA to the nanoprobe targeting loop was then added to nanoprobe sample, at a ratio of 1000:1, and incubated for 2 hours at 37°C, before lifetime decay measurement was repeated.

3.3. Results

3.3.1. Investigating nanorods stability after synthesis and ligand exchange

The reliability of the GNR synthesis procedure was tested in small growth solution volumes of 10 ml with varying concentrations of silver ions. This synthesis method has been found to be reliable; resulting in stable and reproducible GNRs. GNRs were synthesised using the silver assisted seed mediated method. Growth solutions were produced with increasing silver concentrations from 120, 150, 180, 210 or 240 µl of silver nitrate per 10 ml of GNR growth solution and the extinction spectra was measured by UV-Vis spectroscopy (Figure 16a). This resulted in the production of GNRs with an increase in the longitudinal plasmon peak with increasing silver concentrations. This simple change in silver concentration allows the fine control of the peak longitudinal wavelength and the accurate and repeatable synthesis of GNRs. This is important in the nanoprobe design, as it allows continuous synthesis of GNRs with a longitudinal peak that overlaps with the emission spectra of cy5 (Figure 16b). This maximises the energy transfer and fluorescence quenching that occurs between the GNR surface and cy5, resulting in a probe with low background fluorescence.

Gold is not a toxic substance and gold nanospheres alone are thought to be relatively harmless to cells, however, there have been many reports of the toxic effects of GNRs in cells (111). Gold nanorods are made of the same material than gold nanospheres but are coated in a bilayer of CTAB during the synthesis procedure, which is responsible for rod shape formation and stability by preventing aggregation. It is this CTAB coating which is also responsible for the toxic effects, with many reports showing a reduction in cell viability (112)(113). This has resulted in the development of ligand exchange protocols, where the CTAB bilayer is removed and replaced with a biocompatible surface (114). The round-trip phase transfer has been developed, in which GNRs are extracted into an organic phase where CTAB is exchanged for dodecanethiol (DDT). GNRs are then extracted into an aqueous phase where DDT is exchanged for mercaptohexanoic acid (MHA) (99). This ligand exchange process was investigated to determine if stable nanorods with a biocompatible surface could be produced for cell culture applications.

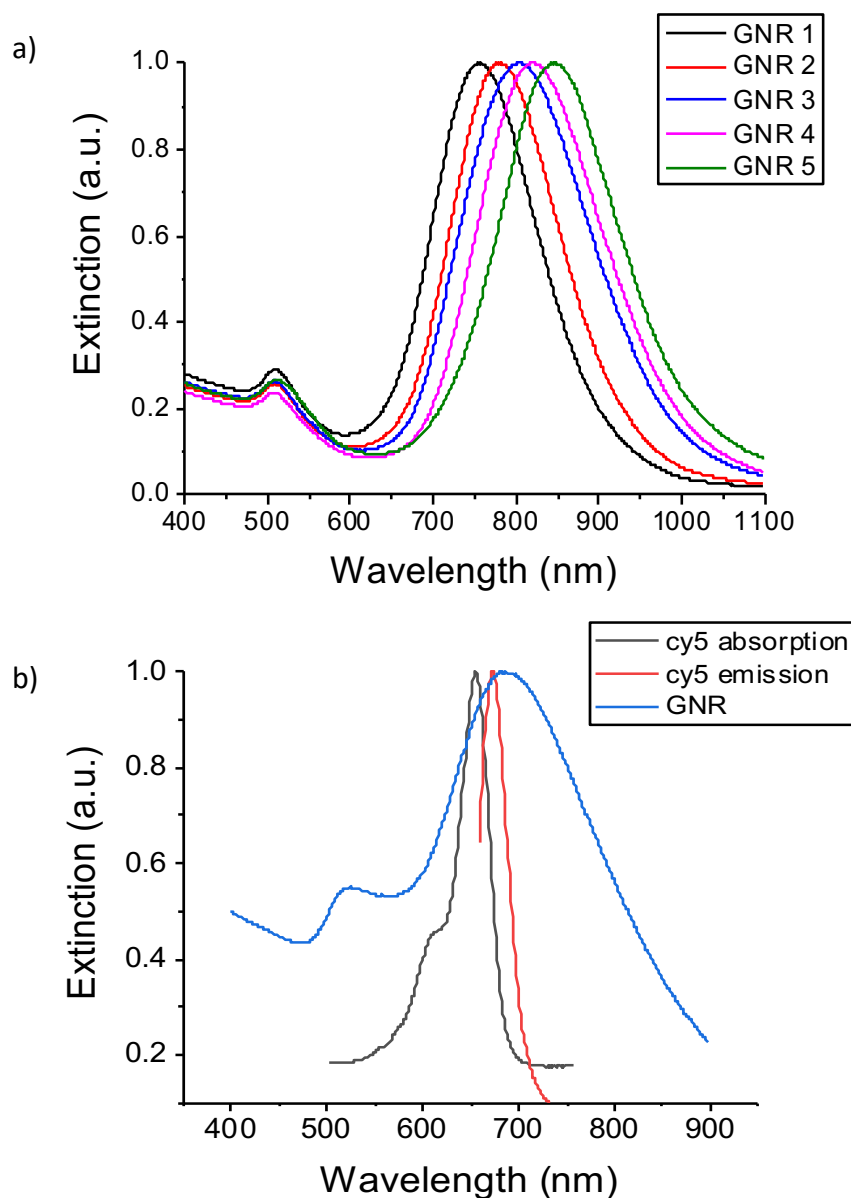


Figure 16. The extinction spectra of GNRs is controlled during synthesis.

a) The longitudinal peak of GNRs can be tuned during synthesis by the concentration of silver ions. The peak longitudinal wavelength increases from sample 1 to 5 by increasing the silver concentration in the nanorod growth solution. b) The wavelength of the GNR longitudinal peak overlapping cy5 emission is essential for the optimal performance of the nanoprobe.

Gold nanorods were synthesised using the seed mediated, silver assisted method to produce stable GNRs coated in CTAB. A sample of GNR-CTAB used to take extinction and zeta potential measurements and the remainder of the sample was processed through the ligand exchange protocol to produce GNRs coated with MHA (GNR-MHA).

The extinction spectra of both GNR-CTAB and GNR-MHA samples demonstrate that the absorbance properties do not greatly change when the surface ligand is changed, which suggests the structure of GNRs is maintained (Figure 17a). Both samples display a transverse peak at ~ 520 nm, which represents the short axis of the GNRs. However, there is a slight blue shift in the longitudinal peak after the ligand exchange process, from 778 nm in GNR-CTAB to 750 nm in GNR-MHA. This blue shift is hypothesised to be due to the heating process of the ligand exchange, which may cause melting of the GNR ends, resulting in a slight shortening of the rods.

To confirm if the ligand exchange process had successfully been achieved, the zeta potential charge of each sample was measured. This can give an indication if a successful exchange has occurred, as CTAB is a positively charged surfactant, while MHA is a negatively charged ligand. Zeta potential measurements were performed on a Zetasizer. Each sample was measured twice and in triplicate and the average value was calculated as the zeta potential charge. The results show a clear change from GNR-CTAB that has a positive charge of +46.6 mV (± 5.8 mV), to GNR-MHA that display a negative charge of -41.3 mV (± 1.8 mV) (Figure 17b). These values are in agreement with measurements in the literature, which reported that CTAB-GNRs have a zeta potential of +45 mV and MHA-GNRs -46 mV (115). This significant change of zeta potential confirms a successful ligand exchange.

The extinction spectra give an indication that rod shape is preserved after the ligand exchange process. In order to confirm this, scanning electron microscopy (SEM) of GNR-CTAB (Figure 18a) and GNR-MHA (Figure 18b) was performed. In order to perform nanorod size analysis, 100 GNR particles were imaged for each sample and

the size of each particle was measured using pixel distance in image j, which was then converted to nanometres according to the scale bar. From the size distribution histograms, GNR-CTAB displayed a wider range of sizes, with a higher number of particles displaying lengths above 45 nm. The average length of GNR-CTAB was found to be 42.6 nm, with a standard deviation of 5.70 nm (Figure 18c). For GNR-MHA, the average size was reduced to 37.8 nm, with a standard deviation of 5.02 nm, after the ligand exchange process (Figure 18 d). The average width of GNRs did not see a significant change, with GNR-CTAB displaying a width of 8.5 nm with a standard deviation of 1.1 nm, and GNR-MHA displaying a width of 7.7 nm with a standard deviation of 0.99 nm. As speculated from the GNR extinction spectra, SEM analysis confirmed that there is a shortening of the average length of nanorods coated in MHA as compared to nanorods coated in CTAB (Figure 18e).

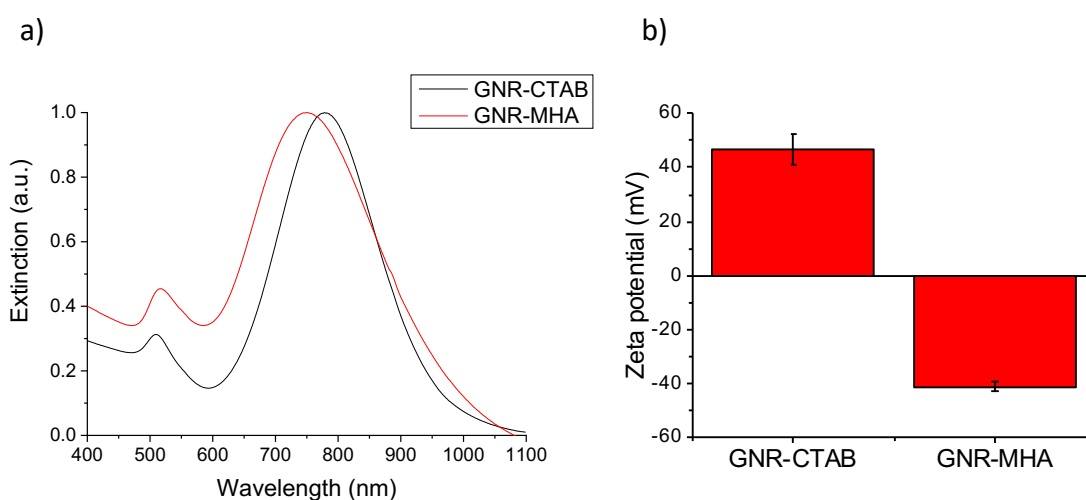


Figure 17. The extinction spectra & zeta potential before & after ligand exchange confirm stability of GNR structure and a successful ligand exchange.

a) The extinction spectra of GNRs coated in CTAB (black) and GNRs coated in MHA (red) demonstrates that the GNRs preserve their structure during ligand exchange as the transverse and longitudinal peaks remain. b) Zeta potential measurements on GNR-CTAB before (left) and MHA after (right) ligand exchange demonstrates the change from a positive CTAB surface to a negative MHA surface charge, indicating a successful ligand exchange.

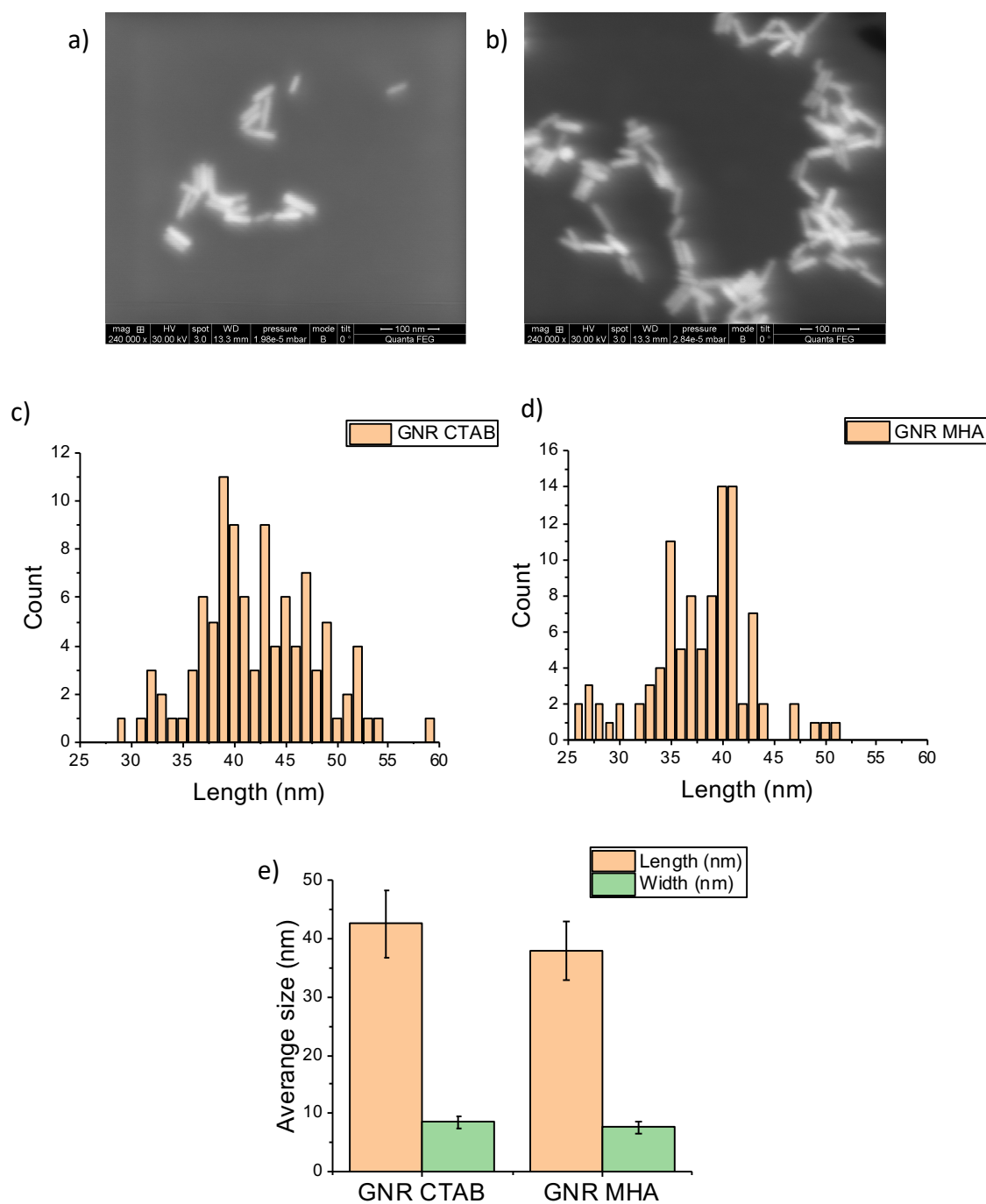


Figure 18. SEM images and size distribution analysis show nanorod shape stability and uniformity.

SEM figures of a) GNR-CTAB and b) GNR-MHA particles show rod shape structures and uniformity. Size analysis of c) GNR-CTAB and d) GNR-MHA shows the size distribution of gold nanorods, with GNR-MHA being shorter than GNR-CTAB. e) The average length and width of GNR-CTAB and GNR-MHA further demonstrates a shortening of gold nanorods after ligand exchange.

3.3.2. Optimisation of DNA loading onto GNRs through varying the pH of salting buffer

Once the stability of GNRs and the success of ligand exchange were confirmed, the next step was to optimise GNR functionalisation with the targeting agent; fluorescently labelled oligonucleotides. A hpDNA was designed against C-MYC mRNA sequence, a cancer oncogene of interest. The C-MYC probe design consists of a 32 bp oligonucleotide that can adopt a hairpin structure due to the eight complementary nucleotides at either extremity. The DNA strand was designed with a disulphide at the 3' and the fluorophore cy5 at the 5'. The design of the probe is stable at room temperature, with the melting temperature at 55.7°C. The binding of the targeting loop to target mRNA ($T_m = 47.6^\circ\text{C}$) was designed to be stronger than the binding of the stem sequence ($T_m = 14.1^\circ\text{C}$), which results in the dissociation of the hpDNA structure upon target recognition.

Previous protocols have used sodium phosphate buffer at pH7 for the salt-aging procedure. However, it was hypothesised that the pH of the salting buffer could be important at facilitating DNA attachment, as the pH also has an effect on the charge of the solution. In order to test the effect of the pH of salting buffer on the probe dynamics, DNA functionalisation was performed with sodium phosphate buffers at pH7, pH6 and pH5. The nanoprobees were made in parallel, with the same concentration of salting buffer, incubation time and washing steps. The final concentration of NaCl added was 125 mM.

Figure 19 shows the fluorescence emission spectra of C-MYC nanoprobees synthesised at pH7 (Figure 19a), pH6 (Figure 19b) and pH5 (Figure 19c) before and after incubation with cDNA. Each sample was measured five times and the average fluorescence intensity was plotted. The peak fluorescence emission was recorded at 665 nm, as this is the peak emission of cy5. The average peak fluorescence and standard deviation (st dev) at the peak value has been reported.

In the nanoprobe synthesised at pH7, the peak fluorescence emission saw an increase after cDNA incubation of 1.35, from 1811.18 (st dev= 104.63) to 2446.25

(st dev= 177.57). In the nanoprobe synthesised at pH6 the peak fluorescence emission increase after cDNA incubation was similar, with a fold change of 1.31, from 2546.93 (st dev= 200.95) to 3357.59 (197.20). The nanoprobe synthesised at pH5 saw the highest increase in peak fluorescence emission following cDNA incubation, with a 1.67-fold change, from 2409.44 (st dev= 79.57) to 4018.99 (st dev= 39.77). These results demonstrate that as the fluorescence intensity of the nanoprobe after cDNA increases, the error of the measurement decreases. Therefore this measurement technique can accurately measure nanoprobes following target DNA incubation in optimal conditions, when DNA opening is highest.

These results suggest that as the pH of the salting solution decreases, the ability of the nanoprobe to detect target DNA increases. This is due to the increase in H^+ ions at lower pH, which will result in an increase in the overall charge of the salting solution. This increase in positive charge reduces the repulsion between the negative DNA and the negative GNR surface, facilitating DNA binding to the GNR surface. These results are in agreement with a study investigating the effect of pH on the attachment of DNA molecules with secondary structures, which found that as the pH increased, the number of DNA molecules bound to the gold surface decreased. This also reported that DNA molecules maintained the secondary structures even at low pH values (116).

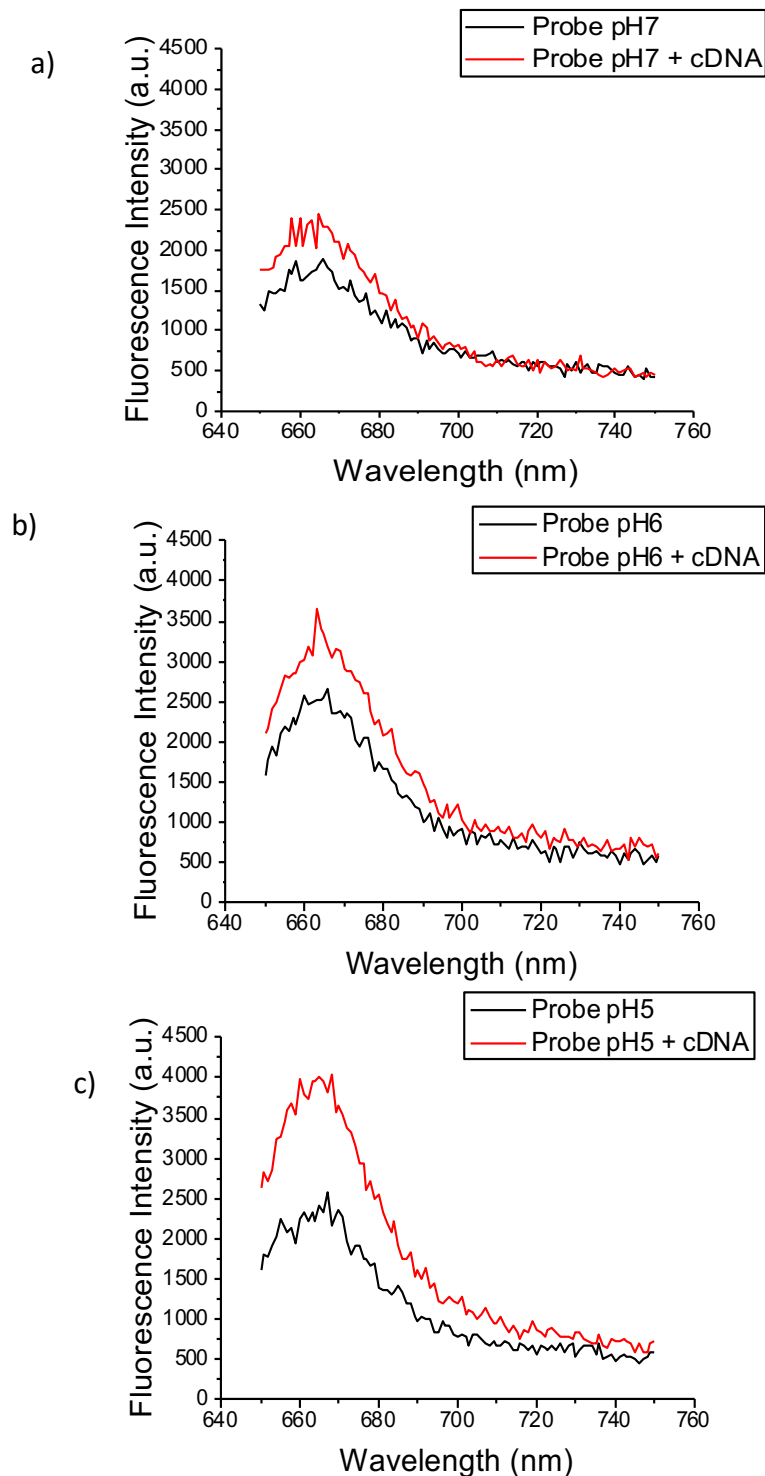


Figure 19. Fluorescence emission change after target DNA incubation is dependent on the pH of salting buffer.

Nanoprobes were synthesised with salting buffer at a) pH7, b) pH6 and c) pH5. The fluorescence emission was measured before (black) and after (red) incubation with complementary DNA. The largest change in peak fluorescence emission was observed with nanoprobes synthesised at pH5, suggesting these nanoprobes have the highest DNA loading and is the most efficient at target recognition.

3.3.3. Investigating specificity of nanoprobe to target DNA through fluorescence spectroscopy

Steady state fluorescence spectroscopy was used to measure the emission intensity of the nanoprobe to determine the background signal of the nanoprobe and binding specificity. Nanoprobe samples were measured before and after incubation with no DNA as a control (Figure 20a), a random DNA sequence (Figure 20b), or a C-MYC cDNA sequence (Figure 20c). The nanoprobe was incubated in an excess of random DNA or cDNA at a molar ratio of 1000 cDNA: 1 nanoprobe for 2 hours at 37°C. The fluorescence emission of each nanoprobe sample was measured before and after incubation five times, and the average intensity was plotted.

In the absence of cDNA the background fluorescence of the nanoprobe is low, with a peak fluorescence of 3203.27 (st dev = 253.23). In the control sample, where no DNA was added, there was almost no change in fluorescence after incubating for 2 hours, with a 1.07-fold increase to 3453.03 (st dev = 577.07). This suggests that the nanoprobe is stable and is not opening randomly. This also demonstrates that the quenching efficiency of the nanoprobe is high in the hpDNA formation. After random DNA incubation there was an increase in fluorescence intensity from 2638.02 (st dev = 258.29) to 3917.38 (st dev = 273.77). This represents a 1.48-fold increase in fluorescence intensity, due to DNA interactions. However, there was a significant increase in fluorescence intensity after C-MYC DNA incubation. Following incubation with target DNA, the nanoprobe fluorescence increased from 2862.93 (st dev = 289.47) to 10,435.77 (st dev = 770.36), which represents a 3.65 fold increase. This indicates that the nanoprobe is selectively binding to the target DNA sequence.

There have been some concerns in the literature over the thermal stability and long-term stability of DNA functionalised nanoparticles. However, using thiol as a DNA anchor has previously been reported to provide the highest thermal stability (103) and these results have shown that the nanoprobe is stable when incubated at 37°C for several hours, suggesting that the hpDNA does not open randomly.

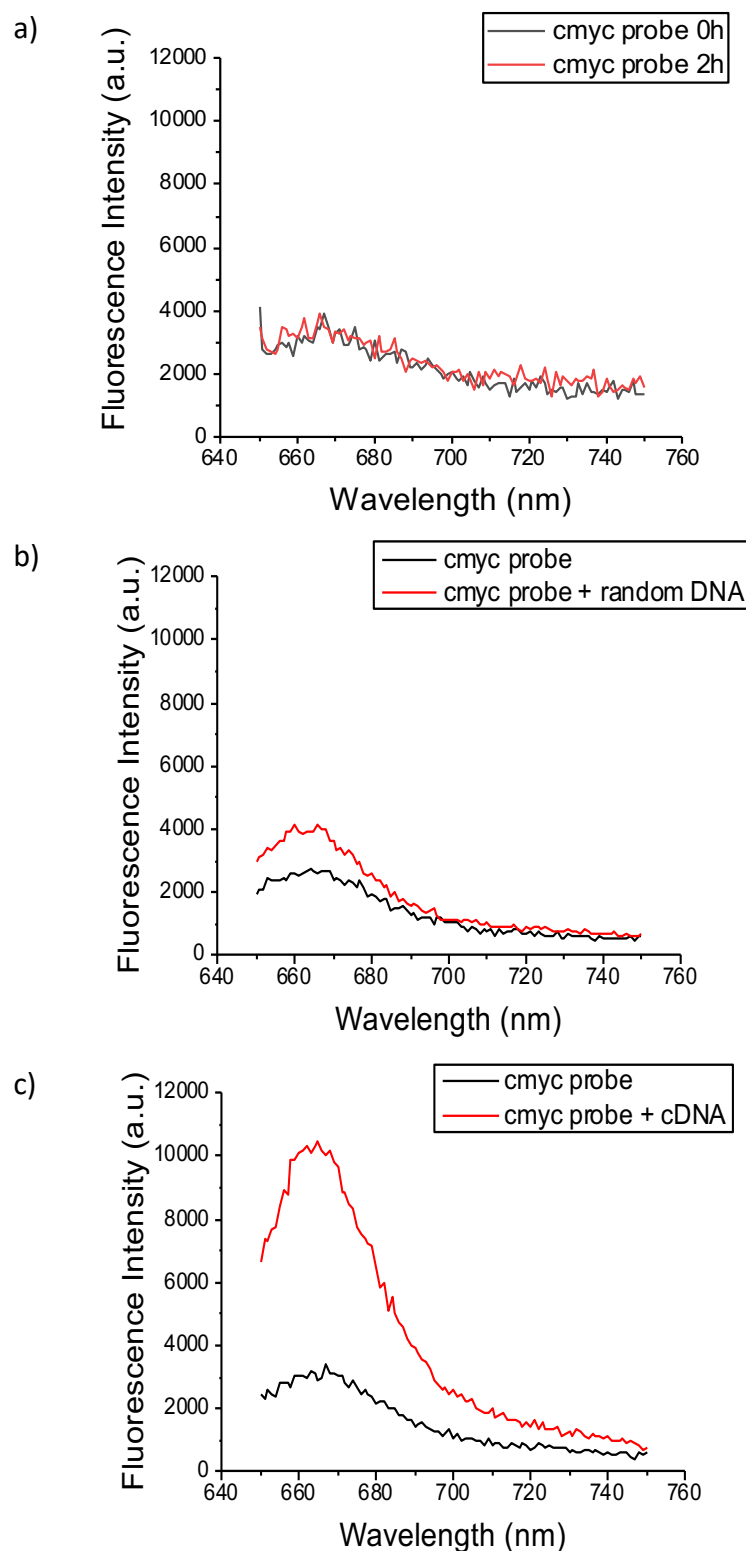


Figure 20. Differential fluorescence emission intensity demonstrates the specificity of nanoprobe binding.

Fluorescence emission of the nanoprobe was taken before (black) and after (red) incubation with a) no DNA b) random DNA and c) complementary DNA. The nanoprobe displayed a significant increase in fluorescence intensity following cDNA incubation, demonstrating binding specificity.

3.3.4. Hybridisation of nanoprobe with targets are both time and target concentration dependent

In order to investigate the fluorescence response of nanoprobes upon incubation with cDNA, the fluorescence emission of C-MYC nanoprobes was measured after incubation with a range of cDNA concentrations (Figure 21a). Each sample was measured in triplicate and an average fluorescence intensity was calculated. Nanoprobe samples were prepared in micro quartz cuvettes at 0.25 nM (OD = 1), and cDNA was added at 0 nM, 10 nM, 25 nM, 50 nM 75 nM and 100 nM. Samples were incubated for 2 hours at 37°C before the emission was measured. The average peak fluorescence emission, which was measured at 665 nm for cy5, was plotted for each sample. These results show a linear trend of fluorescence intensity, from low emission with 0 nM DNA incubation that increases up to 100 nM. A linear fit was applied to the trend with $R^2 = 0.957$.

The fluorescence emission of the C-MYC nanoprobe was also measured over time, from 0 minutes to 120 minutes, following incubation with 100 nM cDNA (Figure 21b). Each sample was measured in triplicate and the average peak intensity was plotted. This showed a linear increase in fluorescence emission up to 60 minutes, after which the fluorescence intensity plateaued and did not increase. A linear fit was applied from 0 to 60 minutes, with the $R^2 = 0.995$. This suggests that when the nanoprobe is incubated with cDNA, target DNA hybridisation starts within the first 15 minutes and takes 60 minutes for the nanoprobe to become saturated, after which time there is no further hpDNA is opened.

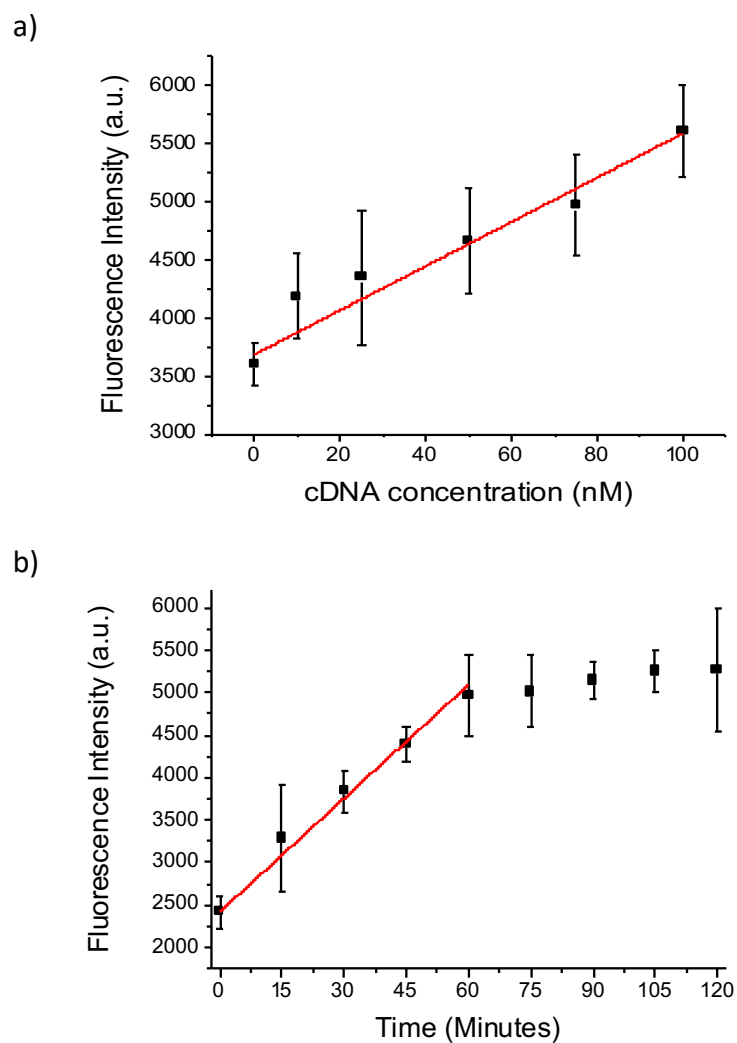


Figure 21. Nanoprobe opening is dependent on the concentration of DNA target and the incubation time.

The peak fluorescence intensity of C-MYC nanoprobe was measured after a) increasing cDNA concentrations from 0 to 100 nM and b) over time after 100 nM of cDNA incubation. The peak fluorescence intensity of the nanoprobe increased linearly with both increasing cDNA concentration and over time, with the highest peak fluorescence occurring after 100 nM of cDNA and fluorescence saturation occurring after 60 minutes of incubation.

3.3.5. Fluorescence lifetime recovery of the nanoprobe after cDNA incubation

Time-resolved fluorescence spectroscopy of C-MYC nanoprobe was measured by TCSPC to investigate the fluorescence decay profiles of nanoprobe following random DNA and cDNA incubation. The fluorescent lifetime of nanoprobe were measured in 10mM Na phosphate buffer pH7. A pulsed light emitting diode of 638 nm was used as the cy5 excitation source at 1 MHz and emission was collected at 665 nm. A diluted sample of GNR-MHA (OD=0.2) was used as the prompt sample, which measures the instrumental response. Time resolved fluorescence spectroscopy measurements were performed on the nanoprobe alone and after a 2-hour incubation alone as a control (Figure 22a), with random DNA (Figure 22b), or with cDNA (Figure 22c) at 37°C.

The analysis of the fluorescence lifetime values, the standard deviation of the lifetimes and the B value contributions can be seen in Table 2. A 3 exponential decay model was applied to each decay profile, as the samples are expected to contain 3 lifetime components; a component from GNRs, quenched cy5 and unquenched cy5. Every decay fit produced χ^2 values close to 1, indicating a good fit of the decay curve. The residual values for each lifetime decay were low ranging between 5 and -5) and the standard deviation, which represents the variation between the experimental data and the fitted decay of each measurement was low for each measurement condition. The analysis of the individual lifetime component features can provide important information on the composition and state of the sample. The B value represents the relative amplitude of the proportion of photons that are contributed from each lifetime component.

The first lifetime component, τ_1 , that ranges from 0.4-0.5 ns, represents the quenched cy5 within the nanoprobe. This is due to the hpDNA configuration of the nanoprobe in the closed state, which localises the cy5 fluorophore close to the GNR surface and results in FRET. τ_2 , the second very short lifetime component of <100 ps represents the gold nanorods, which are not fluorescent but are highly scattering. τ_3 , the third longer lifetime component of ~1.3 ns represents the unquenched cy5.

This longer lifetime exists due to target DNA hybridisation, which opens the hpDNA structure. This results in the cy5 fluorophore no longer being in close proximity to the gold nanorod surface and this removes the fluorescence quenching.

In the nanoprobe control sample, where no DNA was incubated, there is no change in the fluorescent lifetime after 2 hours. In the nanoprobe sample measured alone, the majority of the signal contribution, 90.98%, comes from τ_2 which is the scattering from GNRs, and this remains at 91.46% after a 2-hour incubation alone. The signal from cy5 within the closed hpDNA nanoprobe is low, with τ_1 values of quenched cy5 contributing 3% before incubation and 2% after incubation. The contribution from unquenched cy5, τ_3 , was 6% before and 6.5% after incubation of the nanoprobe alone (Figure 22a). These results show that the majority of the nanoprobe fluorescence lifetime in the closed state comes from the scattering of GNRs, with a low background of cy5 contribution and this does not change following incubation alone, demonstrating the stability of the nanoprobe.

After the nanoprobe was incubated with a random DNA sequence, a slight shift can be seen on the decay profile, indicating a small increase in fluorescence lifetime. This can be seen by a change in the B values for each lifetime component. τ_1 which represents the lifetime of quenched cy5 saw an increase in the contribution value (B1) from 2.86% to 8.91%. τ_2 which represents the scattering of GNRs saw a decrease in the contribution value (B2) from 89.5% to 75.2%. τ_3 which represents the lifetime of unquenched cy5 saw an increase in the contribution (B3) from 7.64% to 15.9% (Figure 22b). Therefore, the overall contribution from cy5 has increased and the contribution from GNRs decreased after random DNA incubation, suggesting that the nanoprobe has some interaction with DNA that is not complementary.

When the nanoprobe was incubated with cDNA, there was a clear and shift in the fluorescence decay profile, indicating a large increase in fluorescence lifetime within the sample, as compared to random DNA incubation. The contribution of τ_1 , which represents quenched cy5 increased from 3.21% before incubation to 8.34% after

cDNA incubation. The contribution of τ_2 , which represents scattering from GNRs, decreased from 90.79% to 70.54% after cDNA incubation. Furthermore, the contribution of τ_3 , which represents the contribution from unquenched cy5, saw a large increase from 6% to 21.12% after cDNA incubation (Figure 22c). This indicates that after cDNA incubation there is a large reduction in the contribution from GNRs and a stronger contribution from unquenched cy5, which results from the dissociation of the hpDNA that increases the distance between the fluorophore and the GNR surface, which removes fluorescence quenching.

To better visualise the lifetime contribution changes in each sample, the B values have been plotted from the nanoprobe control sample, nanoprobe with random DNA and nanoprobe with cDNA (Figure 22d). This shows that in the nanoprobe control sample, the majority of the signal is from gold scattering, with a low background from cy5. After random DNA incubation, the contribution from both quenched and unquenched cy5 slightly increased. When the nanoprobe was incubated with target DNA, the contribution from the GNRs greatly decreased and the contribution from unquenched cy5 saw the highest increase. Therefore, the fluorescence lifetime of cy5 is quenched in the nanoprobe structure and is recovered following cDNA incubation.

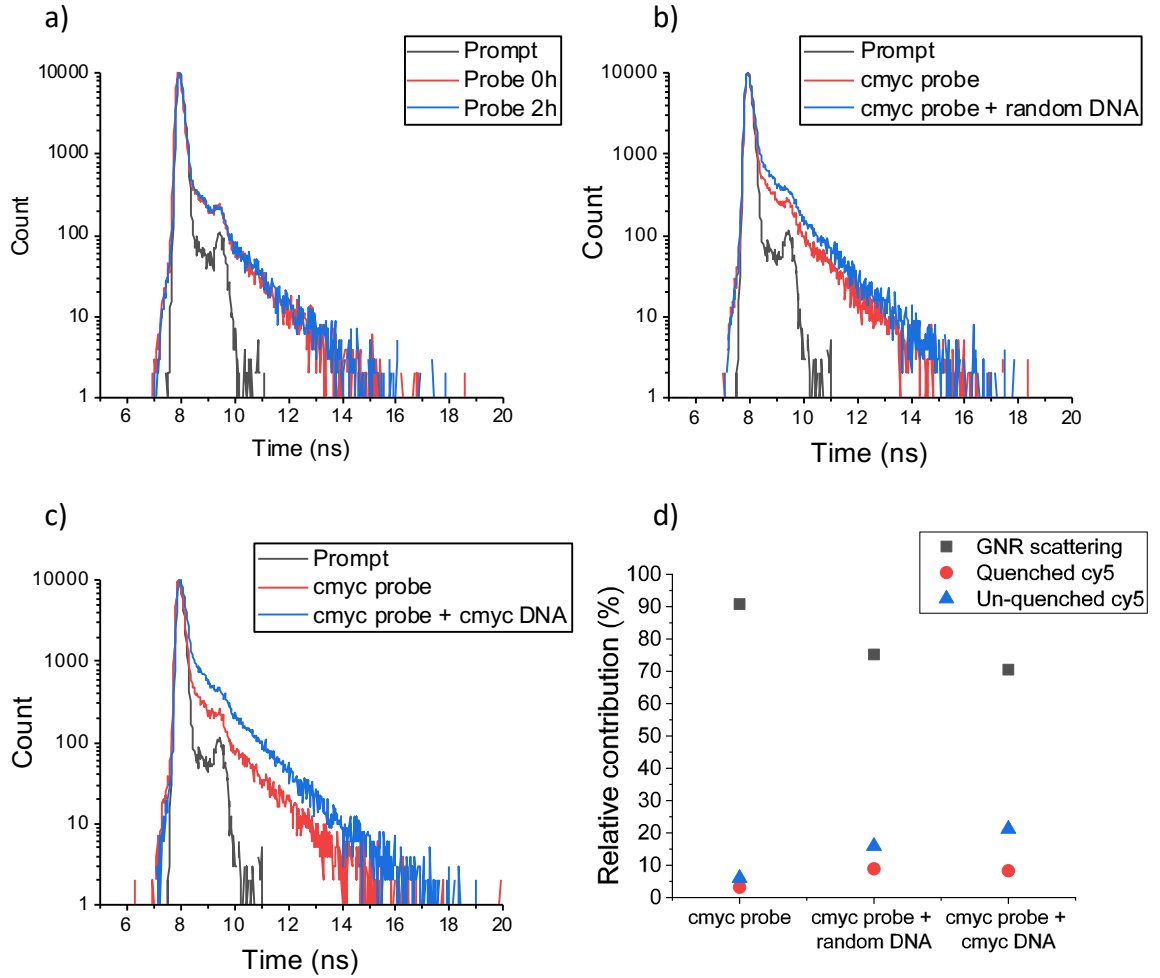


Figure 22. Fluorescence lifetime recovery of cy5 after target DNA incubation.

Fluorescence intensity decay curves of nanoprobes before (red) and after a 2-hour incubation (blue) a) alone as a control, b) after random DNA incubation and c) after complementary DNA incubation. d) The relative contribution of each lifetime component. The fluorescence lifetime of cy5 is quenched in the nanoprobe structure and is recovered after complementary DNA incubation.

Sample	τ_1	B1	τ_2	B2	τ_3	B3	χ^2
C-MYC probe 1	0.55 ns (0.13ns)	3.09	23.1 ps (2.3ps)	90.98	1.31 ns (59ps)	5.94	1.07
C-MYC probe 1 after 2h	0.55 ns (0.16ns)	2.01	22.5 ps (2.8ps)	91.46	1.12 ns (47ps)	6.53	1.01
C-MYC probe 2	0.45 ns (87.9ps)	2.86	19.1 ps (5.2ps)	89.5	1.24 ns (28.3ps)	7.64	1.09
C-MYC probe 2 + random DNA	0.53 ns (0.10ns)	8.91	53.7 ps (5.5ps)	75.2	1.29 ns (36.9ps)	15.9	1.2
C-MYC probe 3	0.38 ns (38ps)	3.21	17.6 ps (4.93ps)	90.79	1.28 ns (27.5ps)	6	0.99
C-MYC probe 3 + C-MYC DNA	0.42 ns (40.3ps)	8.34	44.5 ps (5.87ps)	70.54	1.28 ns (14.1ps)	21.12	0.98

Table 2. Fluorescence lifetime 3-exponential fitting analysis.

A 3-exponential decay fit was applied to each nanoprobe sample before and after a 2-hour incubation alone (probe 1) with random DNA (probe 2) or with complementary DNA (probe 3). Lifetime fitting analysis demonstrates 3 fluorescence lifetime components; 1. quenched cy5, 2. scatter component from gold nanorods 3. un-quenched cy5. Lifetime values (τ) with the standard deviation values in brackets, and the relative contribution values (B) were calculated for each component.

For each steady state and time resolved fluorescence spectroscopy measurements, incubations were performed for 2 hours and 4 hours at 37C°. Results have only been presented for the 2-hour incubation, as this was sufficient time to allow for hpDNA opening in response to target and resulted in the maximum fluorescence response. Incubations performed at 4 hours did not display further fluorescence, suggesting that hpDNA opening was saturated.

Due to the complex nature of the nanoprobe sample preparation, there was limited volume and concentration of each nanoprobe batch produced. Therefore, steady state and time resolved fluorescence spectroscopy measurements were performed once and the remainder of each sample was used for cell work investigations. This is a limitation of each measurement; however consistent results were obtained by repeating experimental conditions with different nanoprobe batches.

3.4. Conclusions

Gold nanorods have been successfully synthesised using the seed-mediated silver assisted growth method. This has resulted in the production of stable GNRs, which can be finely tuned during synthesis to produce the required longitudinal peak for maximum energy transfer with the fluorophore within the nanoprobe. GNRs have been further modified to be biocompatible, through the round-trip ligand exchange process, to produce biocompatible GNRs coated in MHA. Zeta potential and SEM measurements have confirmed the success of the ligand exchange protocol and the stability of GNRs-MHA. These GNRs were then successfully functionalised with fluorophore labelled hpDNA. This was performed through a salt-aging process. The extent of DNA loading using this procedure with a salting buffer at pH7 has previously been shown to be around 110 hpDNAs per GNR (110). This investigation demonstrated that DNA loading is pH dependent, with a higher nanoprobe response at lower pH, therefore the loading density at pH5 may be much higher than this. The pH dependence of DNA attachment was shown by differential fluorescence intensity after incubation with target DNA, with the nanoprobe synthesised at a lower pH value showing the maximum fluorescence response. The discovery that pH value of the salting process has a significant effect on the hpDNA attachment was important to enhance the sensing abilities of the nanoprobe. This has resulted in the production of novel mRNA sensing nanoprobe that have high specificity and fluorescence response to target sequences.

Fluorescence spectroscopy of the nanoprobe in solution phase has been performed to demonstrate the efficiency of fluorescence quenching in the nanoprobe design

and the strong recovery of fluorescence after cDNA incubation. Both steady state and time-resolved fluorescence spectroscopy have demonstrated that the nanoprobe has a low fluorescent background in the closed hpDNA state. The nanoprobe binding specificity has also been demonstrated, with an increase in fluorescence after cDNA incubation, and very little change in fluorescence after random DNA sequence incubation and incubation of the nanoprobe alone. Time resolved fluorescence spectroscopy has demonstrated that the lifetime of cy5 is largely quenched in the closed hpDNA nanoprobe conformation. Following cDNA incubation, the lifetime of cy5 increased as the distance between the fluorophore and GNR surface increases, which removes fluorescence quenching.

The low background fluorescence of the nanoprobe is due to FRET between the fluorophore and the GNR in the closed hpDNA conformation. The energy transfer in the nanoprobe is enhanced due to the surface plasmon resonance of the GNR being tuned to overlap with the emission spectra of cy5 surface plasmon resonance.

However, there are challenges that face the use of nanoprobe for commercial and clinical use. The variability of GNR synthesis is a significant problem that would affect the quenching efficiency, as GNRs produced with a different longitudinal peak may not overlap as strongly with the fluorophore of interest. This was a significant problem at the beginning of this investigation and the process of GNR synthesis was highly optimised to produce narrow and consistent GNR longitudinal peaks. The key factors in GNR synthesis were found to be the temperature of the growth solution incubation, as if the synthesis reaction occurs below 20°C, CTAB forms crystals which limits the reaction and reduces the final concentration of GNRs. Furthermore, the quality of chemicals has a profound effect on GNR synthesis, with impurities in CTAB stocks contributing to a reduction in GNR synthesis concentration and a variability in GNR size, and this is a condition that has been described in the literature (117).

The results in this chapter are significant as they demonstrate that nanoprobe can be designed that exhibit a low initial fluorescence background and display an

increase in fluorescence that is specifically in response to cDNA. This has resulted in the production of C-MYC targeted nanoprobe that are capable of target sequence detection, which demonstrates the ability of the nanoprobe to be used as a fluorescence biosensor.

4. Investigating the uptake and fluorescence of C-MYC mRNA nanoprobe in individual cells using flow cytometry

4.1. Introduction

As discussed in the introduction, current methods of cancer diagnostics are slow, invasive and do not provide individual cell level analysis. The tumour biopsy, a gold standard tumour diagnostic and characterisation technique, involves the removal of a tumour core sample for histological analysis and a diagnostic is made based on cell morphology and protein expression within the extracted tissue (118). As tumours have been shown to be extremely heterogeneous, both within the same tumour mass and between primary and metastatic tumours (119), this bulk analysis can result in the loss of information, by missing genetic cancer subtypes that can have an impact on the treatment decision pathways. Therefore, there is a need for a cancer detection method that is non-invasive, simple, rapid and sensitive to cancer signatures.

A fluorescent GNR nanoprobe has been designed against C-MYC mRNA, which is a cancer biomarker overexpressed in many different cancer types. In this investigation, nanoprobe uptake and nanoprobe fluorescence in individual cells was investigated by flow cytometry. It is important to determine the optimal conditions for nanoprobe uptake, as the ability to detect target mRNA is dependent on nanoprobe internalisation into cells.

Flow cytometry is a powerful measurement technique to detect the scattering and fluorescence properties of individual cells, across large cell populations. It is a rapid and non-invasive procedure that uses lasers to analyse the physical and chemical characteristics of cells. Cells move through a high-pressured stream and pass in single file across a laser beam. As the cell passes through the light, it scatters light in many directions. Forward scatter (FSC) provides information on the size of the cell, as large objects will refract more light, generating a higher FSC signal. Side scatter

(SSC) is dependent on elements within cells, such as mitochondria or granules that can also scatter light. Therefore this measurement can be thought of as a representation of the internal complexity of the cell (80).

Flow cytometry is a widely used technique for analysing the expression of cell surface and intracellular molecules. Developed in the 1960s, flow cytometry has most commonly been used for protein detection within cells, using fluorescently labelled antibodies. Clinical applications of flow cytometry have been rapidly developing, due to the development of smaller, user friendly and less expensive equipment, and they have now become a routine clinical method in hospitals across the world.

Traditional uses of flow cytometry include haematology applications, particularly in antigen detection using antibodies for cell surface protein recognition. The application of flow cytometry in clinical disease diagnostics has been successful in blood cancers. In B cell malignancies, flow cytometry is the common practice technique used to distinguish the aggressive form of the disease from the non-aggressive form (120). These cell types display similar morphologies but have a different expression level of the surface protein CD52 and require different treatment paths. Therefore, flow cytometry is capable of detecting subtle differences in cell expression levels of biomarkers of interest and can facilitate disease state diagnosis to inform on treatment decisions.

Therefore, the clinical value of flow cytometry has been well established for detecting protein biomarkers and cellular changes in blood. The use of flow cytometry for fluorescence detection has been shown to be robust, it can be standardised, and it provides very accurate results. The development of antibody microbeads with different fluorophores of different colours has allowed multiplexed protein biomarker detection (121).

After it was possible to detect protein biomarkers in the blood, interest gained in the ability to detect other blood-based biomarkers. Traditionally, flow cytometry analysis has been limited to proteins and highly expressed DNAs (122). However,

recent technologies have expanded the applications to show that flow cytometry is capable of detecting RNAs expressed in individual cells. One such technique is branched DNA technology, which utilises Z shaped complimentary DNA sequences to bind to target RNA. Multiple Z probes are required to bind to the target RNA, which then initiates binding of an amplification probe that generates the fluorescence signal which is detectable by flow cytometry (123). Commercially available kits using this technology are available, such as PrimeFlow by ThermoFisher (124) which costs £198 for the reagents for one test. ACDBio uses the same technology in RNA scope (125), however the cost for this kit could not be found as the price is quoted individually for each custom made probe. While this is a specific RNA biomarker detection assay it requires cell fixation and permeabilisation in order for the probes to be internalised. Furthermore, for each biomarker target, at least 20 targeting probes are designed to bind along 1 kb of target gene RNA, complicating the probe design process. The assay kits are also very expensive and high costs limit the practicality of using these techniques in clinical practice.

Flow cytometry previously been used to detect GNR scattering and GNR fluorescence within cells. SSC measurements within cells can be informative for cellular uptake measurements, such as the uptake of GNRs, as GNRs have high light scattering abilities. It has been reported that the scattering from a single GNR has the same intensity as the fluorescence from 10^5 fluorescein molecules (126). Fluorescence within a cell can also be measured using flow cytometry. If the laser wavelength is within the excitation peak of the fluorophore, the molecule will be excited, and the emitted photons can be detected.

Flow cytometry has been shown to be a sensitive technique for the fluorescent detection of cancer mRNA biomarkers and cancer cells in a liquid biopsy. Currently available probes are expensive and require complex and lengthy sample treatments. Therefore, a fluorescent nanoprobe that can be added directly to a sample and has the ability to detect cancer mRNAs would be highly advantageous.

An advantage of flow cytometry for mRNA detection is the ability to design different nanoprobe against different mRNA biomarkers, each labelled with a different fluorophore. This would allow multiplexed detection of multiple cancer biomarkers, providing a more robust clinical test than one biomarker would provide.

Furthermore, flow cytometry of a fluorescent nanoprobe is a more efficient and rapid measurement process compared with current biomarker detection techniques. mRNA sensing techniques that are already available involves complex sample preparation and cell transfection for the uptake of the detection agent. Detection of target expressing cells with the nanoprobe requires no sample preparation or transfection reagents, cells are simply incubated with the nanoprobe and then measured after an incubation period.

Compared to the nanoflare design, a nanosensor based on gold nanospheres and short flare DNA recognition sequences, we believe that using GNRs for the nanoparticle core will provide a lower fluorescent background, as they exhibit strong fluorescence quenching abilities due to the overlap of the GNR longitudinal surface plasmon resonance with the fluorophore emission. Furthermore, the sensitivity of detection could be increased as nanoflares have the risk of non-specific release of the DNA flare fragments, while the hpDNA provides more stability as the whole sequence is anchored to the GNR surface through a thiol bond.

The nanoprobe developed in this thesis was initially designed to target C-MYC mRNA. This biomarker was chosen because of the role C-MYC has in cancer development. Cancer is the sequential acquisition of mutations and dysfunctions of key cellular regulation genes. One of these key regulation genes is C-MYC, which normally functions to regulate cell division and cell survival. However, C-MYC has been reported to be overexpressed in over 40% of all human cancers (76). This inappropriate activation promotes tumorigenesis through the uncontrolled replication and survival of cells. Therefore, C-MYC is a common oncogene and provides an attractive target to detect a variety of different cancer cell types.

The objective of this study is to demonstrate GNR uptake into cells through the detection of GNR scattering by flow cytometry. Following GNR uptake studies, selectivity of C-MYC nanoprobe to detect target mRNA expressing cell lines will be demonstrated. Finally, the ability of the nanoprobe to discriminate C-MYC expressing cancer cell lines in co-culture with non-cancer cell lines will be demonstrated. These results support the ability for nanoprobe to detect CTCs in liquid biopsies, by fluorescent flow cytometry measurements.

4.2. Experimental Methods

4.2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) (D5796), non-essential amino acid solution (M7145), sodium pyruvate (S8636), penicillin-streptomycin (P4333) were purchased from Sigma- Aldrich. Fetal bovine serum (FBS) (11550356) and trypsin TrypLE express enzyme (12604013) were purchased from Fisher Scientific. Flow Check Pro Fluorospheres (A63493) was purchased from Beckman Coulter. The Isolate II RNA mini kit, Reverse Transcriptase kit, MyTaq DNA polymerase kit and Isolate II PCR & Gel Kit were purchased from Bioline.

4.2.2. Sample preparation

Cell lines were cultured in DMEM media supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin. For flow cytometry analysis, cells were seeded in a 6-well plate at a density of 500,000 per well and incubated at 37°C in a humidified incubator with 5% CO₂ overnight. The following morning, 0.1 nM of GNRs (GNR-MHA) or nanoprobe (GNR-hpDNA) were added to each well in 1 ml media and incubated for 6 hours. Following incubations, cells were washed with PBS, trypsinised and centrifuged at 1200 rpm for 5 minutes. The cell pellets were resuspended in 1 ml of cold sorting buffer (PBS, 1% FBS, HEPES 25mM). Each sample was then filtered through a 40 µm cell strainer to prevent cell clumping.

4.2.3. Flow cytometry measurement

Flow cytometry measurements were performed on the MoFlo XDP High Performance Cell Sorter, Beckman Coulter. This is equipped with a 366 nm, 488 nm and a 640 nm laser. Before each measurement the lasers were aligned using Flow Check Pro Fluorospheres. Side scatter from the cells was measured with a 488/6 nm laser with a short pass filter of 505nm. The fluorescence from cy5 within the probe was measured with the 640 nm laser with a wideband filter of 670/30. Measurements were recorded using Summit 5.3 software. 10,000 events were measured for each condition before gating the target cell population.

Analysis was performed using Kaluza Flow Analysis Software. For all nanoprobe flow cytometry samples, the fluorescence intensity was normalised by subtracting the control sample fluorescence and normalised by the SSC, which represents GNR uptake.

4.2.4. qRT-PCR

4.2.4.1. RNA extraction

RNA was extracted using Bioline RNA mini kit. 5×10^6 cells were centrifuged for 5 minutes, 1200rpm. The cell pellets were lysed by adding 350 μ l lysis buffer and 3.5 μ l mercaptoethanol, followed by mixing by vortex. Cells were loaded into a collection tube filter and centrifuged for 1 minute. 350 μ l of 70% ethanol was mixed with cell lysate and transferred to RNA mini column. This was centrifuged for 30 seconds. The filter was then transferred to a new collection tube and 350 μ l of membrane desalting buffer was added before centrifuge for 1 minute. 10 μ l of DNase was mixed with 90 μ l of reaction buffer for each sample. 95 μ l of this solution was added to the membrane and incubated at room temperature for 15 minutes. The column then underwent several washing steps. 200 μ l of wash buffer 1, followed by 600 μ l of wash buffer 2 were centrifuged for 30 seconds each. The column was then placed into a new collection tube and a further 250 μ l of wash buffer 2 was added and centrifuged for 2 minutes to dry the membrane. The RNA

was finally eluted by adding 40 µl of RNase free water and centrifuged for 1 minute. The RNA concentration was then measured on a NanoDrop 2000 before being stored at -80°C.

4.2.4.2. Reverse transcription

cDNA was synthesised using the Bioline BioScript Reverse Transcriptase kit. Each RNA sample was diluted to 100ng/µl in RNase free reaction tubes on ice. Each reaction was made up in 20µl following the protocol. Each sample was made with and with reverse transcriptase enzyme and without the reverse transcriptase enzyme, as a control.

The samples were then placed on a hot plate at 45°C for 30 minutes, followed by 85°C for 10 minutes before being kept on ice for immediate use or stored at -20°C.

4.2.4.3. PCR

To amplify the C-MYC gene PCR was performed using the Bioline MyTaq DNA polymerase kit. Forward and reverse primers of 18bp were designed to amplify a 169 bp C-MYC PCR product. The PCR reactions were set up in 25µl final volume. 200ng of cDNA template was added to each reaction. The forward primer had a melting point of 58.5°C and the reverse 58°C. To determine the optimal primer annealing conditions, a temperature gradient was performed between 55°C and 60°C in 0.4°C increments. The PCR programme was as follows on a Bio-Rad DNA Engine, Peltier.

Thermal Cycler:

1. Initial denaturation 95°C for 5 minutes
2. Denaturation 95°C for 1 minute
3. Primer annealing 55°C-60°C for 15 seconds
4. Extension 72°C for 30 seconds
5. Back to stage 2 for 34 cycles
6. Final extension 72°C for 7 minutes

After PCR completion, 5 μ l of each DNA product was loaded on a 3% agarose gel with 1x loading buffer. The gel was run for 30 minutes at 100 V.

4.2.4.4. DNA purification

To remove primer dimers from the PCR product, DNA was purified using Bioline Isolate II PCR & Gel Kit. The DNA sample was ran on a 1.5% agarose gel at 100 V for 30 minutes. The DNA gel slice was then cut out using a scalpel and each slice was weighed. 200 μ l of binding buffer was added per 100 mg of gel. Samples were then placed at 50°C for 10 minutes, vortexing every 3 minutes to dissolve the gel.

Samples were then placed in PCR & gel column and centrifuged 30 seconds 11,000 xg. The columns were washed twice with wash buffer. 20 μ l of elution buffer was added and incubated for 1 minute at room temperature before being centrifuged for 1 minute. DNA was then stored at -20°C.

4.3. Results

4.3.1. Investigating gold nanorod uptake in cell lines

Flow cytometry was performed to study the uptake of GNR-MHA into cells. The optimal concentration of GNR incubation with cells to generate the highest uptake was investigated by varying both the incubation time of GNRs and the concentration of GNRs incubated with cells.

The time dependence of GNR uptake was measured in a non-cancer cell line, Hek-293, and a cervical cancer cell line, HeLa. Cells were left untreated as a control (0 hours) or incubated with 0.1 nM of GNRs for 1, 2, 4, 6 and 8 hours (Figure 23a). After the incubation time was complete, cells were collected and measured by flow cytometry. The SSC of cells was measured with a 488 nm laser and a 505 nm short pass filter. 10,000 events were measured for each condition and the average intensity of the SSC for each cell population was taken. Both cell lines were measured without GNR incubation as a control (0 hours). Both Hek and HeLa cells display a time dependent uptake of GNRs, up to 6 hours. This suggests that at

longer GNR incubations, more GNRs are uptaken into the cells. At the 8-hour time point, the SSC decreased in both cell lines. This could be due to cell division, as the average cell cycle length for Hek cells is 10 hours (127) and HeLa cells is 18 hours (128) and cells within each population will all be at different stages of the cell cycle. Therefore, 8 hours may be too long for GNR incubations, as the cells will start to divide and split the GNR content between 2 new cells. This reduction in SSC after 8 hours could also be due to GNRs being released from the cell, which can occur through uptake through exosome capture, degradation and expulsion from a cell through the lysosome exocytosis pathway (129). Both Hek and HeLa cells display a time dependent uptake of GNRs, with the peak signal occurring at 6 hours.

HeLa cells displayed a higher control SSC as compared to Hek cells, suggesting that the cancer cells are larger and more internally complex than the non-cancer cell line. This difference in initial SSC is important to take into consideration when fluorescence measurements are being taken, as uptake rates of different cells lines could differ and should be normalised to make a comparison. When the SSC was normalised for both cell lines (Figure 23b), it shows that HeLa cells alone do have an initial higher SSC and have an overall higher uptake of GNRs, as compared to Hek cells. Hek cells showed an increase in SSC of 151% after 6 hours, while HeLa cells showed an increase of 184%.

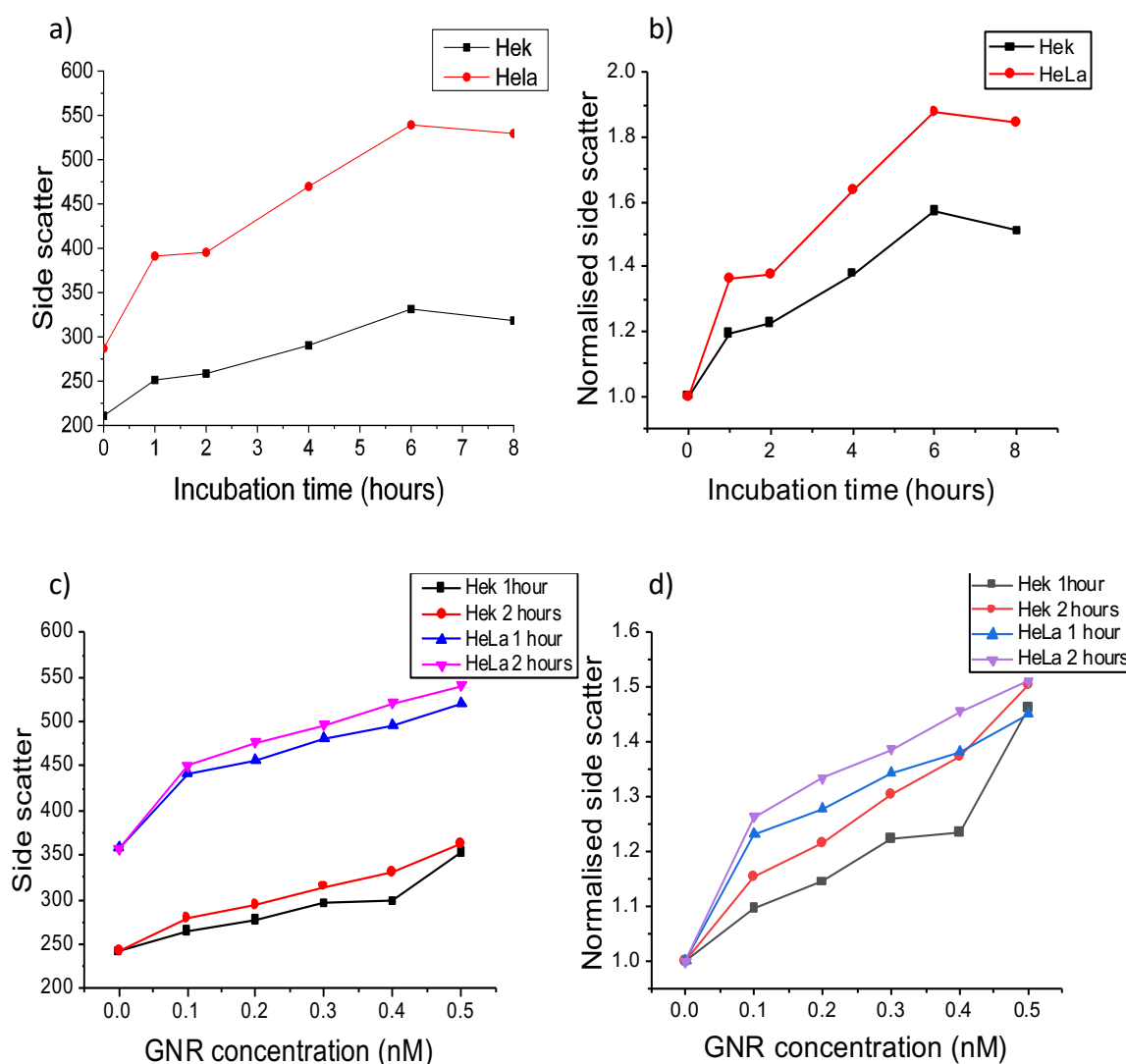


Figure 23. Flow cytometry demonstrates a time and dose dependent uptake of gold nanorods in cell lines.

Flow cytometry was used to measure SSC of Hek & HeLa cells. a) SSC values and b) normalized SSC values after 0.1nM of GNRs were incubated with Hek (black) and HeLa (red) cells from 0-8hours. c) SSC values and d) normalised SSC values after 0-0.5 nM of GNRs were incubated in Hek cells for 1 hour (black) or 2 hours (red) and HeLa after 1 hour (blue) and 2 hours (pink). GNR uptake increased with increasing time, up to 6 hours, and with increasing dosage of GNR incubation. Maximum uptake was seen after 0.1 nM of GNRs for 6 hours or 0.5 nM of GNRs for 2 hours.

The concentration of GNR incubations was also varied, to investigate whether this had an effect on GNR uptake. Hek and HeLa cells were incubated with 0, 0.1, 0.2, 0.3, 0.4 or 0.5 nM of GNRs. Each concentration of GNRs was incubated for 1 or 2 hours (Figure 23c). Both cell lines displayed an increase in SSC for each increasing concentration of GNRs, with a maximum uptake at 0.5 nM. The SSC values were normalised for each cell line and this showed that there was a slight increase in SSC at the 2-hour incubation but there was not a significant difference (Figure 23d). Furthermore, there was not a difference in SSC at 0.5 nM between cell lines. In Hek cells, from 0 to 0.5 nM incubation, the SSC increased by 146% for 1 hour and 150% for 2-hour incubations. From 0-0.5 nM in HeLa cells, there was an increase in SSC of 145% for 1-hour incubation and 151% for 2-hour incubation.

Therefore, maximum GNR uptake was found after incubation with either 0.1 nM for 6 hours or 0.5 nM for 2 hours. For all future flow cytometry experiments, cells were treated with 0.1 nM of GNRs or nanoprobe for 6 hours.

4.3.2. Investigating nanoprobe synthesis conditions on fluorescence in cell lines

Fluorescence spectroscopy of nanoprobe in solution phase demonstrated that the nanoprobe synthesis conditions had an effect on the fluorescence intensity of nanoprobe following incubation with cDNA (Figure 19). Using the same synthesis conditions of varying the pH of the salting buffer at pH7, pH6 and pH5, the fluorescence of nanoprobe was investigated in cell lines by flow cytometry. This was to investigate measuring the fluorescence emission of nanoprobe within individual cells.

C-MYC nanoprobe were synthesised with salting buffers at pH7, pH6 and pH5. 0.1 nM of nanoprobe were incubated with a non-cancer cell line, Hek, and a cancer cell, HeLa, for 6 hours before the fluorescence was measured by flow cytometry (Figure 24a). For each cell line a control sample of untreated cells, a GNR (GNR-MHA) incubated sample without fluorophore and a nanoprobe (GNR-hpDNA) incubated sample were prepared for each salting condition. The cy5 within the

nanoprobe design was excited with a 640 nm laser and emission was collected with a wideband filter of 670/30.

Figure 24a displays the flow cytometry results of cells treated with nanoprobe made with salting buffers at a different pH. Control samples, cells alone with no gold, exhibited very low fluorescence, with Hek control cells displaying an intensity of 0.05 and HeLa cells displaying an intensity of 0.08. Similarly, in cells incubated with GNRs alone, Hek and HeLa cells displayed low fluorescence intensities of 0.06 and 0.11, respectively. In samples incubated with nanoprobe, Hek cells displayed low and similar background fluorescence intensities of 2.74, 3.0, and 2.95 when incubated with nanoprobe synthesised at pH7, pH6 and pH5, respectively. The fluorescence from HeLa cells was dependent on the pH of the salting buffer, with fluorescence intensities of 6.93, 10.97 and 13.16 at pH7, pH6 and pH5, respectively. The maximum fluorescence intensity in HeLa cells was observed with nanoprobe that were synthesised with salting buffer pH5. Therefore, there was an observed increase in fluorescence intensity as the pH of the salting buffer decreased. This suggests that DNA functionalisation is maximum at pH5, as increasing DNA loading would provide more hpDNA targeting loops to detect target mRNA. This results in a higher fluorescence intensity being observed as there are more hpDNA targeting loops to bind to the target mRNA and produce a fluorescent signal.

The effect of final salt concentration in the salt-aging process was also investigated on the performance of the nanoprobe by flow cytometry. The standard salt aging protocol states that the volume of salting buffer added should be 1/5 of the total volume of a 5 nM GNR sample. To investigate if increasing the salting buffer above this volume would increase the DNA loading and therefore increase the fluorescence intensity of the nanoprobe, two nanoprobe samples were made; one nanoprobe sample was made with normal salting buffer volume up to 100%, and the second sample had a higher salting buffer volume of 150% (Figure 24b).

Flow cytometry was performed in two cell lines to analyse the fluorescence of nanoprobe synthesised with different salting buffer volumes. Hek was analysed as

a non-cancer cell line and compared with a prostate cancer cell line, PC3. For each cell line, a control sample and a GNR treated sample were prepared alongside nanoprobe incubations. 0.1 nM of GNRs and nanoprobes were incubated with each cell line for 6 hours.

The control and GNR cell samples displayed a low fluorescence intensity of 0.1 for Hek and 0.19 for PC3. In nanoprobe treated Hek cells, both nanoprobes displayed low background fluorescence intensity of 2.80 for 100% salting and 2.62 for 150% salting. In PC3 cells treated with the nanoprobe that was synthesised with 100% salting buffer, the fluorescence intensity was 81.62, which shows an increase of 29-fold, as compared with Hek cells. PC3 cells that had been incubated with the nanoprobe synthesised with 150% salting buffer displayed a fluorescence intensity of 86.4, which is a 33-fold increase in fluorescence intensity, as compared to Hek cells incubated with the same nanoprobe.

The significant increase in fluorescence intensity for both nanoprobes in PC3 cells demonstrates the nanoprobe ability to specifically fluoresce in response to binding to C-MYC mRNA. This analysis showed that the nanoprobe synthesised with 150% salting buffer had a higher increase in the fluorescence intensity, as compared to the nanoprobe synthesised at 100% salting. However, the 150% salting buffer nanoprobe sample had a much lower final nanoprobe concentration after the salt-aging process, as compared to the 100% salting nanoprobe, due to GNR aggregation. This may be due to a too high concentration of Na⁺ ions that caused the negatively charged GNRs to irreversibly aggregate. Therefore, increasing the volume of salting buffer does slightly increase the DNA loading onto some GNRs, resulting in an increased fluorescence intensity in cells, but it also causes the aggregation of GNRs and loss of sample volume. Therefore, 100% salting buffer at pH5 was used for all future nanoprobe samples, as this produced stable GNR samples that have a high density of fluorophore labelled hpDNA attachment.

The low background fluorescence of the nanoprobe observed in non-cancer cells, as compared to the high fluorescence intensity in cancer cells, demonstrates the

ability of the nanoprobe to distinguish C-MYC expressing cancer cell lines. The background fluorescence in the non-cancer cell line could be due to inefficient quenching in the probe design or due to a low expression level of C-MYC mRNA, which has been investigated by PCR and will be discussed.

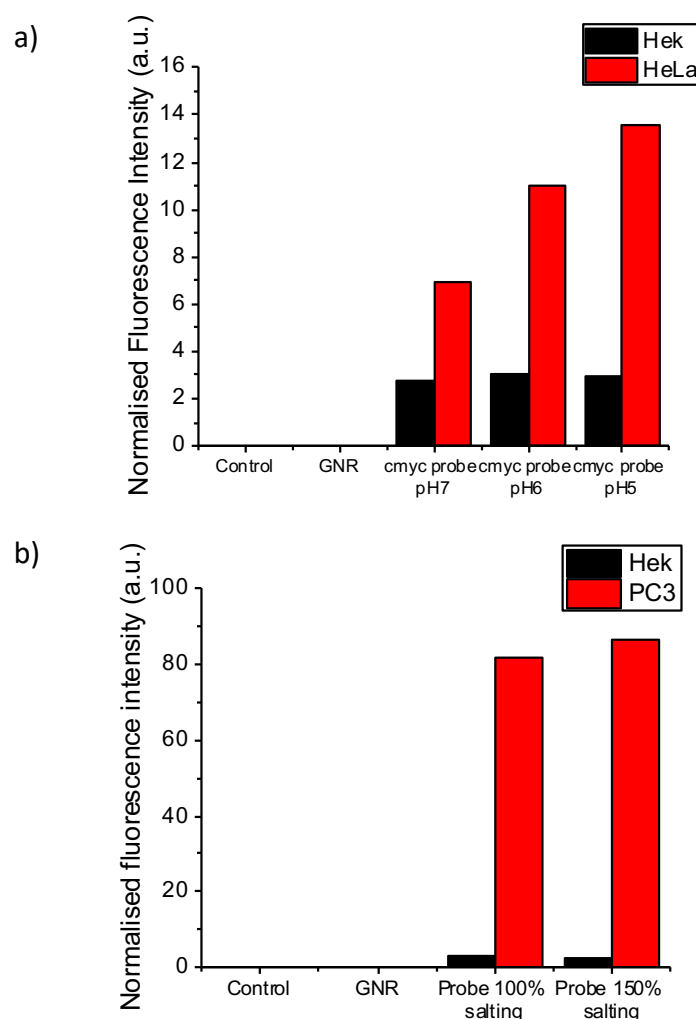


Figure 24. Investigating salting condition on the fluorescence intensity of the nanoprobe in cells.

a) Flow cytometry was performed on Hek and HeLa control cells, GNR treated cells and cells treated with C-MYC nanoprobe synthesised with a salt-aging process at pH7, pH6 or pH5. The fluorescence background was low in Hek cells, while differential fluorescence intensity was observed in HeLa cancer cells. The highest fluorescence intensity was observed after incubation with the nanoprobe synthesised at pH5, indicating maximum DNA loading. b) The extent of salt-aging on the nanoprobe fluorescence of was investigated by synthesising c-Myc nanoprobe with 100% total salt-aging and 150% salt aging. Hek and PC3 cells were measured alone as control, after gold nanorod incubation or after c-Myc nanoprobe synthesised with 100% or 150% salt buffer concentration. There was not a significant difference in fluorescence intensity between each nanoprobe, indicating increasing salt concentration does not improve the nanoprobe detection ability.

4.3.3. Investigating nanoprobe sensitivity to C-MYC expression levels in cell lines

The nanoprobe has been shown to be able to distinguish cancer cell lines from non-cancer cell lines. To determine whether the fluorescence intensity of the nanoprobe reflects the expression levels of C-MYC, real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) of C-MYC mRNA levels was performed and compared to flow cytometry fluorescence intensity with the C-MYC nanoprobe (Figure 25). qRT-PCR is a technique to measure RNA expression levels. mRNA was extracted from cell lines and reverse transcribed to produce complement DNA. C-MYC DNA was then amplified through the use of C-MYC primers and the amount of C-MYC product was quantified through the fluorescence intensity of a fluorophore that emits only when the C-MYC gene was amplified. The expression level of C-MYC mRNA in different cancer cell lines was then compared to the non-cancer cell line Hek, which was used as a reference. The cancer cell lines that were investigated were ovarian cancer cells A2780, cervical cancer cells HeLa and prostate cancer cells PC3. Flow cytometry fluorescence measurements was performed with the same cell lines following incubation with C-MYC nanoprobe.

qRT-PCR of C-MYC mRNA demonstrated that each cell line had a different C-MYC mRNA expression. Hek was used as a reference and set to 1, with A2780 cells showing a 1.58 increase in expression, HeLa cells showed a 2.36 increase in expression and PC3 cells showed the highest C-MYC expression with a 21.5 fold-change increase (Figure 25a). Flow cytometry of the same cell lines incubated with the C-MYC nanoprobe displayed the same trend of fluorescence (Figure 25b). Hek displayed the lowest fluorescence intensity of 0.89, followed by A2780 at 1.64, then HeLa at 2.69 and then with the highest fluorescence intensity PC3 at 12.2. In flow cytometry analysis, PC3 cells displayed a 13.7-fold change in fluorescence as compared to Hek cells.

These results show that the nanoprobe is capable of detecting target mRNA within cells and the fluorescence intensity of the nanoprobe in flow cytometry is proportional to the expression levels of target mRNA. In comparison, a recent

report demonstrated that the fluorescence intensities measured from five different nanoflares did not correlate to mRNA expression levels that were assessed by qRT-PCR (130).

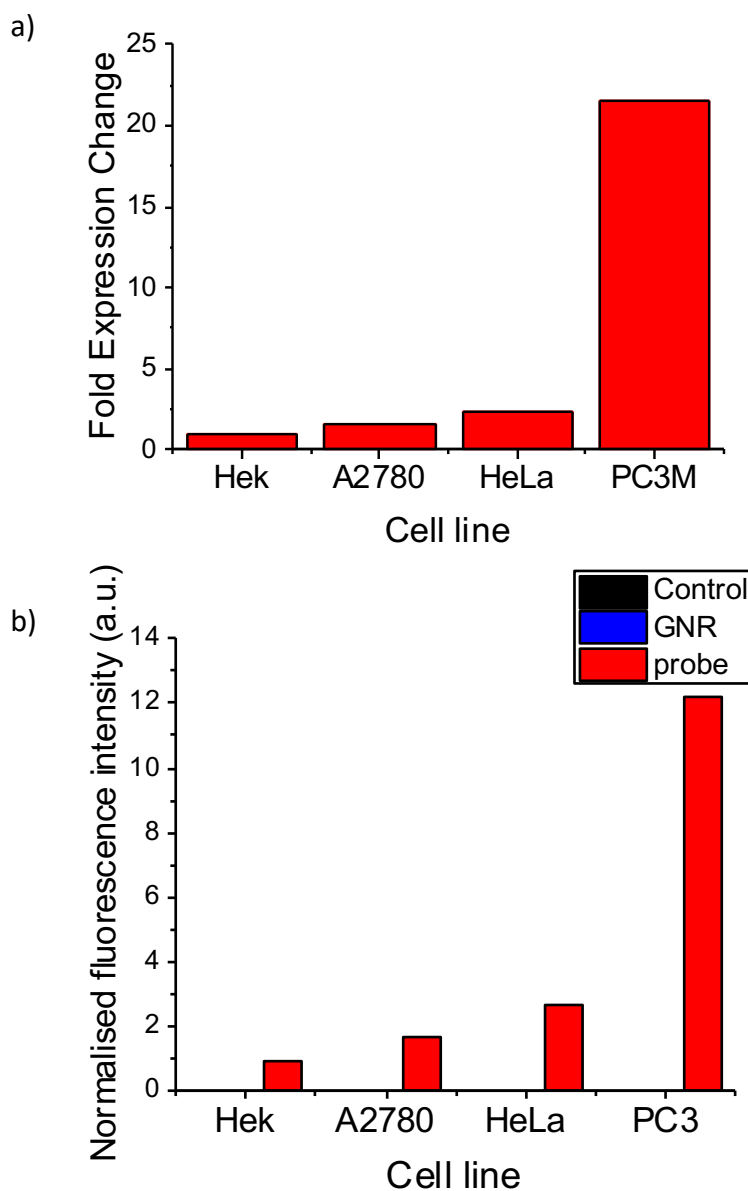


Figure 25. Detecting differential C-MYC mRNA expression in cell lines by qRT-PCR and flow cytometry of the C-MYC nanoprobe.

C-MYC mRNA expression levels were measured in Hek, A2780, HeLa and PC3 cell lines using a) qRT-PCR and b) flow cytometry after C-MYC nanoprobe incubation. This demonstrates that different cell lines have different C-MYC mRNA expression levels and the nanoprobe fluorescence intensity is correlated to target expression levels.

4.3.4. Investigating the dependence of nanoprobe fluorescence on hpDNA dynamics

To further confirm whether the fluorescence intensity measured by flow cytometry represented nanoprobe hpDNA opening, Hek and PC3 cells were incubated with C-MYC nanoprobe with either nanoprobe or nanoprobe pre-treated with excessive cDNA (nanoprobe-cDNA) (Figure 26). Nanoprobes alone were incubated with cell lines as described previously. Nanoprobes were also prepared by incubating the nanoprobe with cDNA for 4 hours before incubating with cells. The significant increase in fluorescence intensity of the nanoprobe after cDNA incubation, as shown in Figure 26a, indicates the opening of hpDNA of the nanoprobe following cDNA incubation.

Cells were then incubated with either nanoprobe or nanoprobe-cDNA and the fluorescence intensity was measured by flow cytometry. As seen previously, both control and GNR treated cells displayed low background fluorescence intensity, with Hek control cells displaying a value of 0.05 and Hek GNR cells displaying a value of 0.06. In cells incubated with nanoprobe alone, Hek cells displayed low fluorescence intensity of 0.95 while PC3 cells displayed a fluorescence intensity of 6.65, which shows nanoprobe in PC3 cells displayed a 7-fold increase in fluorescence. When the same cells were incubated with nanoprobe-cDNA, the fluorescence intensity of Hek cells increased dramatically to 6.6, which matched the fluorescence of PC3 cells incubated with nanoprobe alone. In PC3 cells incubated with nanoprobe-cDNA, there was a slight increase in fluorescence intensity to 8.1, as compared to PC3 cells with closed hpDNA (Figure 26b). These results further support the notion that the hpDNA is not opening randomly within cells, but the fluorescence intensity is directly related to the amount of target mRNA, which results in nanoprobe binding, DNA opening and removal of fluorescence quenching.

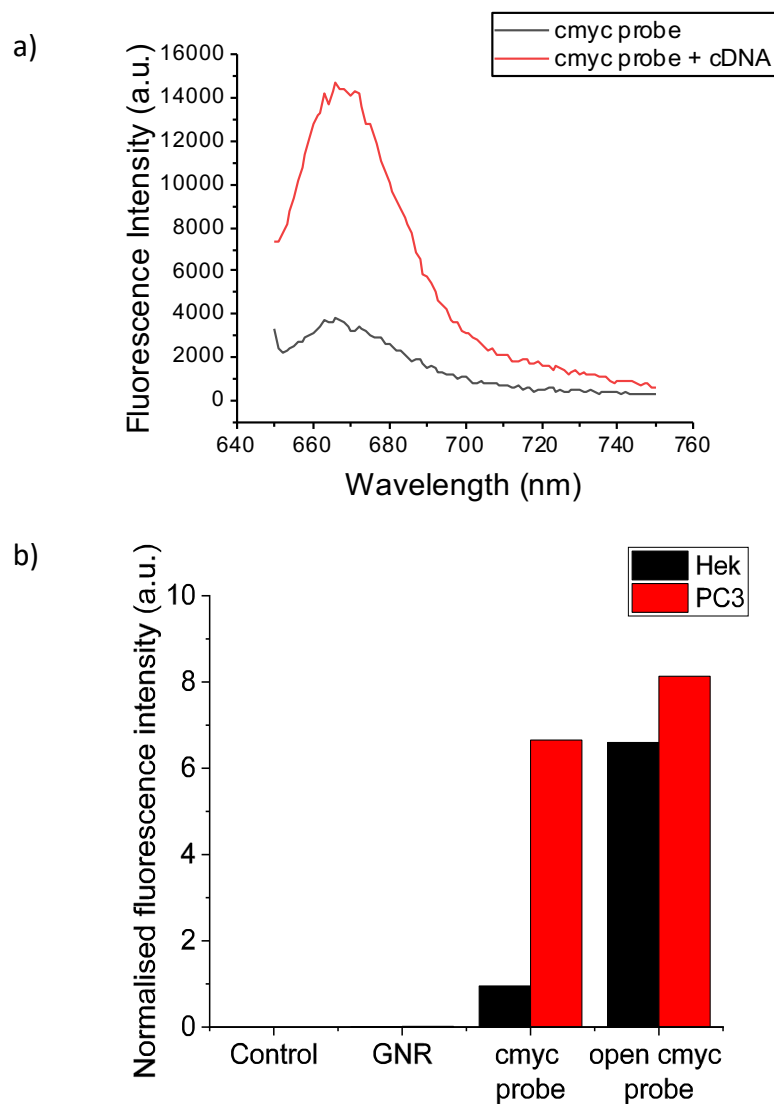


Figure 26. Nanoprobe fluorescence is dependent on hpDNA opening dynamics.

a) The fluorescence emission of C-MYC nanoprobes alone (black) and C-MYC nanoprobes incubated with cDNA (red) was measured. b) Hek and PC3 cells were analysed by flow cytometry with control cells, cells treated with GNRs and cells treated with nanoprobe alone or with nanoprobe-cDNA. The fluorescence intensity of the nanoprobe alone demonstrates the preferential nanoprobe hpDNA opening in PC3 cells. The fluorescence intensity of cells incubated with nanoprobe-cDNA is high in both cell lines.

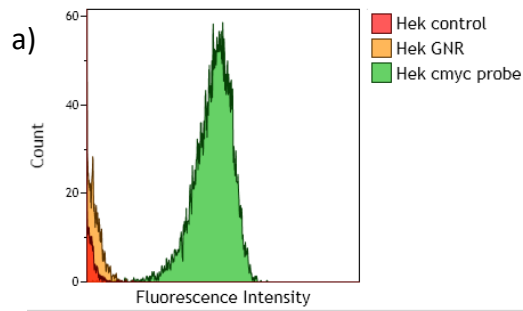
4.3.5. Detecting cancer cells in co-culture

To determine if C-MYC expressing cancer cells could be identified when mixed in a background of non-cancer cells, nanoprobe treated PC3 cells were diluted in nanoprobe treated Hek cells at ratios of 1:10, 1:100 and 1:1000 and the fluorescence intensity was measured by flow cytometry. Hek and PC3 cells were incubated separately with 0.1nM of nanoprobe for 6 hours. Cells were then collected, counted and diluted to the same concentration of cells per ml. PC3 cells were then diluted with Hek cells in the desired ratios. Three samples were prepared for each dilution and flow cytometry measurements performed on each sample for 10,000 counts. Figure 27 displays the histogram and dot graph for one sample for each dilution.

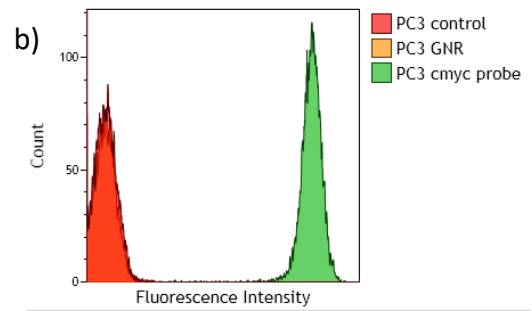
Comparing Hek and HeLa cells alone, there is a clear difference in fluorescence intensity, with Hek nanoprobe cells displaying a fluorescence intensity of 7.88 and PC3 nanoprobe cells displaying a fluorescence intensity of 204.83. Therefore, PC3 nanoprobe treated cells displayed a 26-fold increase compared to Hek nanoprobe cells (Figure 27a&b). This difference is maintained when PC3 are diluted in Hek cells at ratios 1:10, 1:100 and 1:1000. Histograms of fluorescence intensity of mixed samples show the Hek cell population (gate A) and the PC3 cell population (gate B) can be clearly distinguished by fluorescence intensity (Figure 27c-e). For each dilution condition, two fluorescent peaks can be visualized, demonstrating that Hek nanoprobe and PC3 nanoprobe cells can be clearly distinguished. For the 1-10 dilution, Hek cells displayed an average intensity of 8.55 (st dev = 0.017) and PC3 cells displayed an average of 205.17 (st dev = 2.11). For the 1-100 dilution, Hek cells displayed an average intensity of 8.57 (st dev = 0.086) and PC3 cells displayed an average of 212.51 (st dev = 4.76). For the 1-1000 dilution, Hek cells displayed an average intensity of 8.50 (st dev = 0.087) and PC3 cells displayed an average of 195.09 (st dev = 30.98). Furthermore, this separation can also be visualized on a cell dot plot, where each measurement is plotted by its SSC and fluorescence intensity (Figure 27f-h). This shows Hek cells display a lower SSC and an average fluorescence

of 8.52 as compared to PC3 cells, which display a higher SSC and fluorescence, with an average fluorescence of 211.11. Furthermore, when the cell populations are gated individually, Hek was gated A and PC3 gated B, the total number of PC3 cells present in each dilution sample is an accurate reflection of the dilution factor.

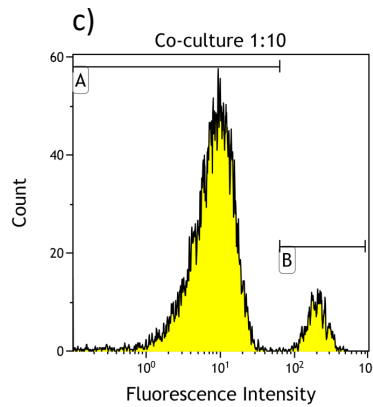
These results demonstrate that the nanoprobe is capable of detecting C-MYC expressing cancer cells diluted in a sample of non-cancer cells, which mimics the detection of CTCs in liquid biopsies. Previously described methods of circulating mRNA biomarker detection through PCR and genome sequencing techniques cannot be used on live cells and cannot provide individual cell level analysis. They involve complex, lengthy and expensive sample processing techniques that can provide false results through contamination or technical errors. Furthermore, this bulk analysis loses information on rare mRNA expressing cells and cannot detect rare expressing mRNAs cell-to-cell variation. Therefore, nanoprobe detection by flow cytometry offers the potential for a rapid and simple detection method for circulating mRNA biomarkers within CTCs.



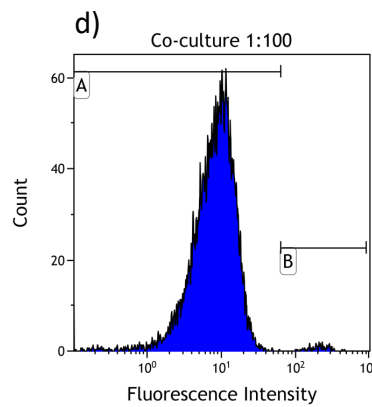
Sample	Total Cell Number	Fluorescent Intensity
Hek control	8,715	0.1
Hek GNR	8,457	0.1
Hek probe	8,365	7.87



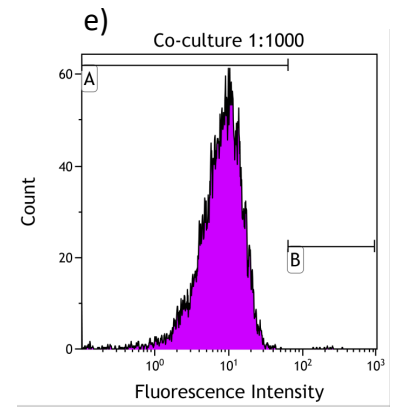
Sample	Total Cell Number	Fluorescent Intensity
PC3 control	8,621	0.19
PC3 GNR	8,505	0.19
PC3 probe	9,035	204.83



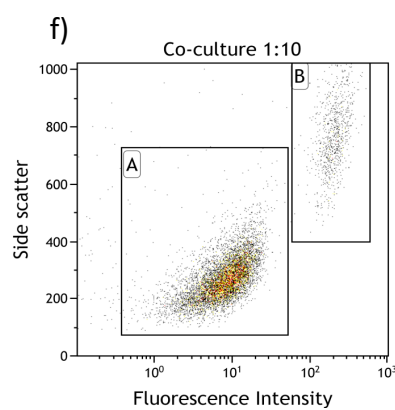
Gate	Total Cell Number	Fluorescent Intensity
A	7,941	8.41
B	849	203.76



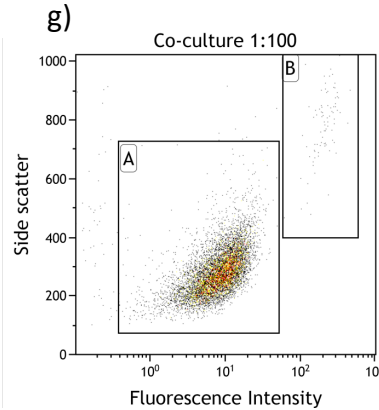
Gate	Total Cell Number	Fluorescent Intensity
A	8,610	8.56
B	86	214.10



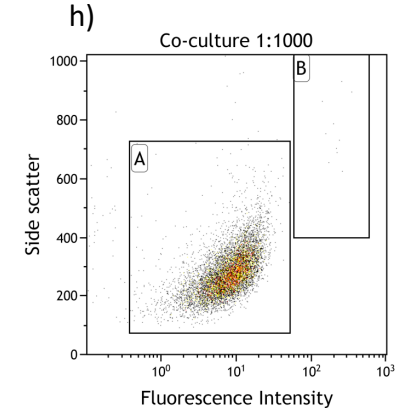
Gate	Total Cell Number	Fluorescent Intensity
A	8,648	8.45
B	10	217.98



Gate	Total Cell Number	Fluorescent Intensity
A	7,849	8.47
B	850	203.66



Gate	Total Cell Number	Fluorescent Intensity
A	8,544	8.61
B	87	211.7



Gate	Total Cell Number	Fluorescent Intensity
A	8,590	8.49
B	10	217.98

Figure 27. C-MYC nanoprobe can selectively detect C-MYC expressing cancer cells in co-culture.

Flow cytometry was used to measure the fluorescence intensity of control, 0.1 nM GNR and 0.1 nM nanoprobe treated cells in a) Hek and b) PC3 cells. Hek cells treated with nanoprobe display some background fluorescence, with a clear fluorescence signal in nanoprobe treated PC3 cells. Histograms of cell count against fluorescence intensity after PC3 cells were diluted in Hek cells at ratios of c) 1:10 d) 1:100 and e) 1:1000. Hek cells were selected under gate A and PC3 cells under gate B. The cell number and fluorescence intensity show PC3 can still be detected at 1:1000 dilution. Cell plots of side scatter vs fluorescence intensity of the same sample dilutions of f) 1:10, g) 1:100 and h) 1:1000 shows the difference in the cell populations based on both SSC and fluorescence intensity.

As the dilution of PC3 cells in Hek cells was repeated in triplicate and resulted in very similar fluorescent intensity values, as demonstrated by low standard deviations, we can have confidence in the reliability of flow cytometry to distinguish nanoprobe positive and nanoprobe negative samples. For each measurement, 10,000 events were measured, and an average fluorescence intensity was recorded. A valuable parameter for these measurements would be the coefficient of variation (CV) value, which measured the uncertainty of a flow cytometry measurement. This can add extra information that is not provided by standard deviation, as the CV value is dimensionless and does not depend on the intensity of the measured population, therefore it can be used to compare the variation between different sample populations. The CV value represents the percentage of data spread, from the mean value of the population.

4.4. Conclusions

Flow cytometry has been shown to be a powerful tool to measure GNR uptake within cell lines. GNR uptake was detected by measuring the SSC of cells incubated with GNRs and uptake was found to be both dose and time dependent. The SSC values increased with increasing GNR concentrations, suggesting that the GNR internalisation increased with higher incubation concentrations. Normalisation of SSC from cancer and non-cancer cell lines demonstrated that different cell lines with different initial SSC values had similar GNR uptake rates. Furthermore, the time of GNR incubation was investigated, with maximum SSC values found after a 6-hour incubation. This is important to determine the maximum uptake of GNRs, as the ability to detect target mRNA is dependent on the nanoprobe internalisation and making contact with mRNA molecules. It is important to find an optimal incubation condition as if the nanoprobe is intended for use in cancer diagnostics, such as the detection of CTCs in a patient blood sample, a short incubation time would be preferential for speed and efficiency of the measurement. The maximum GNR uptake was found after incubation with 0.1 nM GNRs for 6 hours or with 0-0.5 nM of GNRs for 2 hours.

Flow cytometry has also been demonstrated as a powerful measurement technique to detect C-MYC nanoprobe fluorescence within cells. Following nanoprobe incubation, the fluorescence intensity from individual cells was measured. The fluorescence intensity detected by flow cytometry has been demonstrated to correlate to the expression levels of target mRNA, when compared to qRT-PCR expression levels for different cell lines. The fluorescence intensity of the nanoprobe in non-cancer Hek cells that display low expression of the target mRNA was low, demonstrating the nanoprobe does not open randomly within cells, and the fluorescence intensity demonstrated a significant increase in PC3 cells, which have a high expression of target mRNA. Furthermore, the nanoprobe has been shown to be capable of detecting target mRNA expressing cancer cells, when diluted in co-culture with non-cancer low target expression cells. This provides

evidence that the fluorescence intensity of the nanoprobe is proportional to the expression levels of the target mRNA. The ability of nanoprobe to reliably and sensitively detect mRNA expression levels within single cells is a powerful measurement technique for the analysis of complex liquid biopsies. Therefore, nanoprobe provide an enhanced ability to not only detect biomarker mRNAs but to compare expression levels between samples.

This is the first time that fluorescent hpDNA functionalised nanoprobe have been used to detect intracellular mRNA expression by flow cytometry. These results support the ability of GNRs to be internalised into cells and to detect and discriminate biomarker mRNA expressing cells. The use of flow cytometry allows the rapid and simple detection method to measure the fluorescence intensity of the nanoprobe. These results support the use of nanoprobe for the detection of rare mRNA biomarker expressing CTCs within a liquid biopsy.

5. Designing Nanoprobes against multiple mRNA biomarkers and mutational P53

5.1. Introduction

5.1.1. Probe design

In order to have a functional and stable nanoprobe, it is important to understand the design process and how these factors affect the nanoprobe performance. The stability of a hpDNA structure is greatly dependent on the composition of the sequence and the sequence length. The DNA melting temperature (T_m) is the temperature at which 50% of a double stranded DNA sequence becomes dissociated. The melting temperature is influenced by the nucleotide composition, specifically the G-C content of the DNA sequence. Complementary binding between DNA is facilitated by hydrogen bonds. The binding between adenine and thymine bases forms two hydrogen bonds whereas the binding between guanine and cytosine forms three hydrogen bonds. This extra bond in the G-C pairing requires more energy to break; therefore, DNA with a higher G-C content will have a higher melting temperature. The melting temperature of DNA is also influenced on the length of the sequence. The longer the DNA length, the more bonds needs to be broken therefore the higher the energy required (131).

The design process begins with selecting a biomarker of interest and designing the recognition element, the hpDNA targeting loop, to be complimentary and specific to this target. In comparison with the nanoflare design, the nanoprobe hpDNA design could provide enhanced mRNA detection and specify. The nanoflares reported have a recognition sequence that varies between 12 to 15 bp long (71). The targeting loop of the hpDNA within the nanoprobe have sequence lengths between 16 to 21 bp. Reports on DNA probes have shown that the optimal length of the target recognition sequence is between 15-30 nucleotides. Therefore, nanoprobes will provide an enhanced mRNA binding selectivity over nanoflares.

The sequence and length of the hairpin stem is also very important. The stem sequence binding must be strong enough to maintain the hpDNA structure but not too strong, as the stem binding must dissociate upon target binding. Stem stability can be enhanced by increasing the G-C content, which will increase the binding strength and produce a higher signal to background ratio. Probes with a stable stem are also beneficial as they will demonstrate higher selectivity, as the stem binding will only be dissociated after binding to a sequence that is of higher energy than the stem binding. The number of nucleotides in the stem design also had an effect on FRET between the fluorophore at the 5' of the DNA and the GNR surface, as the stem and loop must be long enough to remove fluorescence quenching when the hpDNA is opened.

Previous studies within the group have compared different stem sequences and lengths, to find the optimal design for the nanoprobe. This found that an 8-nucleotide sequence with 50% GC content provided sufficient stability in the closed state and the ability for nanoprobe opening (132).

Spacer nucleotides are also introduced into hpDNA designs to increase the distance between the targeting loop and the gold surface, with the aim to create more space for target binding and prevent crowding of this region. A previous study investigating the optimal spacer sequence and composition found that a spacer of two adenine nucleotides (AA), resulted in the nanoprobe with the highest stability. These spacer nucleotides increased the attachment of hpDNA to the gold surface, which resulted in nanoprobe with a low fluorescence background and the highest fluorescence response when incubated with target DNA (8).

The length per base in single stranded DNA has been calculated to be 0.67 nm (133), therefore the AA spacer will introduce a 1.34 nm gap between the cy5 and GNR when the hpDNA is in the closed state. When the nanoprobe is opened in the C-MYC nanoprobe design, which contains 35 nucleotides, this will result in a distance of 23.45 nm between cy5 and the GNR.

5.1.2. Selection of cancer biomarkers

New nanoprobe were synthesised to target other cancer mRNA biomarkers of interest; lung cancer biomarkers SOX2 and HER1, and a breast cancer biomarker HER2.

Lung cancer is the leading cause of cancer deaths worldwide, accounting for more deaths than breast, prostate, pancreatic and colon cancer combined (134). Unlike many other cancers, survival rates have not seen a great improvement in the last 40 years.

A lung cancer biomarker of interest is the transcription factor Sry-related HMG box 2 (SOX2). The normal function of SOX2 involves the regulation of key steps in embryogenesis, such as maintaining the self-renewal, multi-differentiation and reprogramming of stem cells. However, dysregulation or inappropriate expression of SOX2 has been implicated in tumour growth, drug resistance and metastasis (135). In the majority of cancers, this is due to an increased expression or gene amplification of SOX2.

SOX2 overexpression has been reported in every subtype of lung cancer. This has been confirmed clinically from DNA sequencing of lung cancer patient samples, (136). In an imaging study, SOX2 was found to be overexpressed in 77% of patients with non-small cell lung cancer (137). SOX2 has been reported to be overexpressed in 20% of patients with lung adenocarcinoma, 60% of patients with squamous cell carcinoma (138). The mechanisms by which SOX2 expression promote cancer growth are not fully understood, however it is known that SOX2 levels increase as the cancer progresses. Therefore, a nanoprobe to detect the overexpression of SOX2 could be valuable for lung cancer screening and monitoring.

Another lung cancer biomarker of interest is the human epidermal growth factor receptor 1 (HER1, or also known as EGFR). When activated, this receptor promotes cell growth, proliferation and survival. The amplification of this gene results in the

over expression of this receptor, which leads to uncontrollable cell growth and survival signals and tumorigenesis (139).

The identification of the roles of HER1 in lung cancer lead to the development of HER1 targeting drugs, such as gefitinib, erlotinib, afatinib, brigatinib and icotinib (140). These drugs were designed to inhibit the growth receptor and prevent tumour growth, and while they demonstrated disease regression in some patients, the success of these drugs has been limited due to toxicity and drug resistance (141)(142).

Breast cancer is another prevalent cancer type with over 55,000 new diagnoses and 12,000 deaths every year in the UK (143). A breast cancer biomarker of interest is human epidermal growth factor receptor 2 (HER2). This gene is in the same family as HER1, and functions in the same way to regulate the normal growth and survival of cells. In breast cancers, HER2 gene amplifications have been associated with promoting tumour growth and survival. Around 25% of all breast cancers show overexpression of HER2 and this is associated with a more aggressive subtype of breast cancer (144). Both mRNA and protein levels have been shown to be elevated in HER2 positive patient samples (145). In routine diagnostics of breast cancer, a tumour biopsy is analysed by a pathologist and given a HER2+ or HER2- score based on the fluorescence intensity of the sample staining. The HER2 status of a patient is an important factor for the treatment pathway, as they are more likely to respond to HER2 targeting drugs, rather than general chemotherapy (146). However, the current scoring method is objective, and studies have shown the scoring system can differ in different hospitals, resulting in false results (147). Therefore, a non-invasive method to detect the presence of cancer and determine the genetic profile of the tumour would be highly beneficial.

The identification of cancer biomarkers has opened new opportunities for cancer diagnostics techniques. Personalised cancer therapies have had limited success due to the heterogeneity and resistance of tumours at late stages and current diagnostic techniques are not robust. If overexpressed cancer biomarkers can be used for early

detection, then combining detection with personalised medicine at early disease stages could greatly improve the success of treatment (148). Furthermore, combining these cancer biomarkers will greatly improve the sensitivity of the diagnostic technique.

As well as designing nanoprobes to detect overexpressed cancer biomarkers, there is also great interest in developing nanoprobes that can detect mutant cancer biomarkers. One mutational biomarker of interest is the tumour protein 53 (P53). P53 is a tumour suppressor gene, which has been named the “guardian of the genome” (149) due to its range of functions in regulating cell proliferation and survival. In healthy cells, P53 is activated in response to cell stress signals, such as DNA damage. It then activates a cascade of cellular pathways, such as cell-cycle arrest or controlled cell death, apoptosis, to prevent the division and survival of damaged cells (150) (Figure 28). Therefore, P53 has important roles as a tumour suppressor.

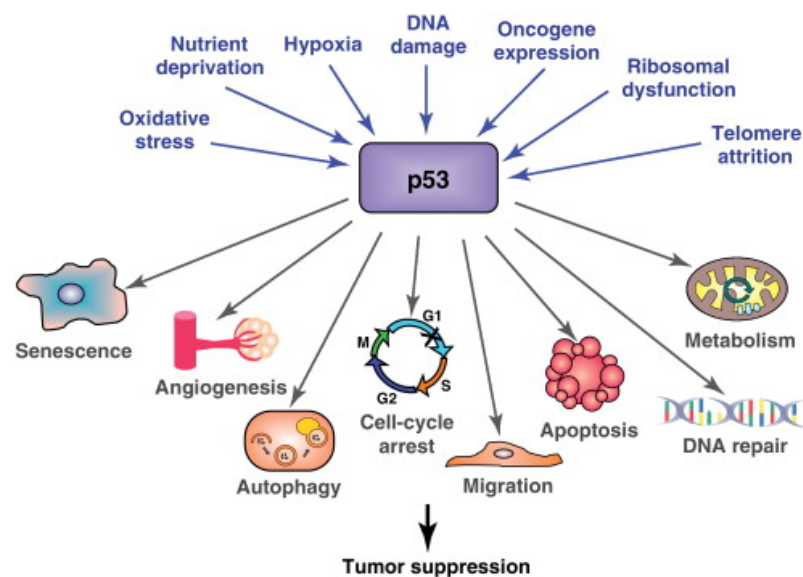


Figure 28. The regulation network of P53 in response to cellular stress signals.

P53 acts as a tumour suppressor by activating cell regulation pathways in responses to cell stress, such as DNA damage. Image was used with permission from Trends in Cell Biology, Copyright © 2011.

The role of P53 in cancer is not due to overexpression of the gene but is due to mutations in P53 that inhibit its anti-tumour functions. It is one of the most commonly mutated genes in cancer, being reported to be mutated in over 40% of all human cancers (151). Furthermore, it has been reported to be a driver of cancer formation, with mutations detectable at early stages of lung cancer and detectable in plasma (152,153) making it an attractive biomarker for early cancer detection. A rapid detection method of P53 mutation signatures would have widespread cancer diagnostics applications.

Previous studies utilising gold nanospheres with fluorophore labelled hpDNA has shown that there is a difference in target binding when one nucleotide mismatch is introduced (154). Therefore, it is possible to design a nanoprobe DNA sequence that is capable of discriminating a single nucleotide mutant biomarker sequence from the wildtype sequence.

This chapter will demonstrate the design and synthesis of nanoprobe against multiple cancer biomarkers. Nanoprobes were synthesised to target the overexpression cancer biomarkers SOX2, HER1 and HER2. The design process of each nanoprobe is described and the importance of DNA designs is discussed. The fluorescence response of each nanoprobe was investigated by fluorescence spectroscopy after c DNA incubation in solution phase and in target expressing cell lines by flow cytometry.

Nanoprobes were then designed against a P53 wildtype and mutation sequence. The ability of the nanoprobe to discriminate a one-nucleotide mismatch was investigated by fluorescence spectroscopy and flow cytometry, and the positioning of the mismatch sequence was investigated. This investigation will show that stable nanoprobe can be synthesised against a range of target mRNA biomarkers. Furthermore, nanoprobe are capable of detecting a target sequence with a fluorescence intensity double than that of a single mismatch mutation.

5.2. Sample preparation

5.2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) (D5796), non-essential amino acid solution (M7145), sodium pyruvate (S8636), penicillin-streptomycin (P4333) were purchased from Sigma- Aldrich. Fetal bovine serum (FBS) (11550356) and trypsin TrypLE express enzyme (12604013) were purchased from Fisher Scientific. Flow Check Pro Fluorospheres (A63493) was purchased from Beckman Coulter.

5.2.2. Steady state fluorescence spectroscopy

The fluorescence emission spectrum was measured using a Fluorolog-3 spectrofluorometer with excitation at 638 nm and a 5 nm slit. The measurement range was set from 650 nm to 750 nm to collect the emission from cy5. Each sample measurement was repeated 5 times and averaged to obtain an emission intensity graph. Measurements were taken with nanoprobe alone and then 2 hours after incubation with either complementary DNA or one nucleotide mismatch DNA, at 1000:1 ratio.

5.2.3. Flow cytometry

Cell lines were cultured in DMEM media supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin. For flow cytometry analysis, cells were seeded in a 6 well plate at a density of 500,000 per well and incubated at 37°C in a humidified incubator with 5% CO₂ overnight. The following morning, 0.1 nM of GNR-MHA or nanoprobe was added to each well in 1 ml media and incubated for 6 hours. Following GNR incubations, cells were washed with PBS, trypsinised and centrifuged at 1,200 rpm for 5 minutes. The cell pellets were resuspended in 1 ml of cold sorting buffer (PBS, 1% FBS, HEPES 25mM). Each sample was then filtered through a 40 µm cell strainer to prevent cell clumping. Flow cytometry measurements were performed on the MoFlo XDP High Performance Cell Sorter, Beckman Coulter. This is equipped with a 366 nm, 488 nm and a 640 nm laser. Before each measurement the lasers were aligned using Flow

Check Pro Fluorospheres. Side scatter from the cells was measured with a 488/6 nm laser with a short pass filter of 505nm. The fluorescence from cy5 within the probe was measured with the 640 nm laser with a wideband filter of 670/30. Measurements were recorded using Summit 5.3 software. 10,000 events were measured for each condition before gating the target cell population.

Analysis was performed using Kaluza Flow Analysis Software. For all nanoprobe flow cytometry samples, the fluorescence intensity was normalised by subtracting the control sample fluorescence and normalised by the SSC, which represents GNR uptake.

5.3. Results

5.3.1. Nanoprobe design

Nanoprobes have been designed against multiple different cancer biomarker genes of interest. The hpDNA probe sequences were all designed and validated in the same way. First, potential target mRNA sequences were identified by performing a literature review of existing gene target probes. In the case of C-MYC the sequence used was identified by an existing FISH probe, whereas HER1, HER2 and SOX2 were identified through existing PCR primers (155)(156)(157). The P53 probes were custom designed by identifying a hot spot mutation region, at codon position 175, from the P53 database (158). Several steps were then taken to validate the probe sequence and specificity. The NCBI gene database (159) was used to search for the gene of interest, with the search set up specifically for the *Homo sapiens* gene variant. This provides information such as the gene location, expression and length. The gene DNA sequence was then accessed through NCBI reference sequences with GenBank. This provided the full DNA sequence length and gene features. The RNA sequence from the target gene was then opened, which is the current known or predicted transcripts of the gene.

Once the RNA sequence was obtained the target region of interest, which is complimentary to the probe targeting sequence, can be identified by using the

“Find in this Sequence” feature. This was used to validate the existence of the target sequence in the mRNA of interest and it can also determine the position of the target. Exons are coding regions of mRNA, so it is important the target existed in an exon region and not a non-coding region of mRNA. For each sequence identified, the RNA sequence was identified and the target position in an exon confirmed. Below is the information from each target sequence validation.

C-MYC

NCBI Gene: MYC proto-oncogene, bHLH transcription factor [Homo sapiens (human)] (160)

RNA sequence: Homo sapiens MYC proto-oncogene, bHLH transcription factor (MYC), transcript variant 1, mRNA (161)

Located on chromosome 8, 4,518 bp linear mRNA, 3 exons total

Target sequence position: 1212-1228 on exon 2

HER1

NCBI Gene: EGFR epidermal growth factor receptor [Homo sapiens (human)] (162)

RNA sequence: Homo sapiens epidermal growth factor receptor (EGFR), transcript variant 1, mRNA (163)

Located on chromosome 7, 6369 bp linear mRNA, 28 exons total

Target sequence position: 866-883 on exon 5

HER2

NCBI Gene: ERBB2 erb-b2 receptor tyrosine kinase 2 [Homo sapiens (human)] (164)

RNA sequence: Homo sapiens erb-b2 receptor tyrosine kinase 2 (ERBB2), transcript variant 1, mRNA (165)

Located on chromosome 17, 4,664 bp linear mRNA, 27 exons total

Target sequence position: 2623-2640 on exon 20

SOX2

NCBI Gene: SOX2 SRY-box 2 [Homo sapiens (human)] (166)

RNA sequence: Homo sapiens SRY-box 2 (SOX2), mRNA (167)

Located on chromosome 3, 2520 bp linear mRNA, 1 exon total

Target sequence position: 444-464 on exon 1

TP53

NCBI Gene: TP53 tumor protein p53 [Homo sapiens (human)] (168)

RNA sequence: Homo sapiens tumor protein p53 (TP53), transcript variant 1, mRNA (169)

Located on chromosome 17, 2591 bp linear mRNA, 11 exons total

Target sequence position: 717-732 on exon 5

Finally, a basic local alignment search tool (BLAST) (170) search was performed to confirm specificity to the target mRNA. BLAST is an algorithm that allows the comparison of a reference sequence to all known DNA, RNA and protein sequences for the comparison of similar or complementary sequences (171). A nucleotide megablast was performed on the targeted mRNA sequence within the human genomic plus transcript database, which includes both DNA and RNA sequences. A sequence was only chosen for the probe design if it displayed 100% recognition to the target gene and did not display 100% recognition to any other gene.

Table 3 contains all of the probe sequences, along with the structural information on the probe design. The total probe length varied from 34 bp long to 39 bp long, with the targeting DNA loop ranging from 16 bp to 21 bp long. The same stem sequence was used for each probe design, which consists of 8 nucleotides. This has been shown to be an optimal length in previous studies, as it is long enough to maintain a stable hairpin structure but can be dissociated upon target loop binding (132). If the stem sequence is too long, the strength of the stem binding may be stronger than the binding of the targeting loop, which would prevent the dissociation of the hairpin upon target binding.

When the hpDNA exists at temperatures below its melting temperature, it exists in a closed state and can spontaneously bind to its target. The melting temperature of each probe design was calculated using the Integrated DNA Technologies

OligoAnalyzer (172). Each probe design displayed a melting temperature of around 55°C, indicating that the probe will be stable at room temperature and 37°C.

If the melting temperature of the loop to target binding is higher than the melting temperature of the stem, the stem can be disassociated upon target binding and the hpDNA structure opens. For each probe design, the melting temperature for the loop sequence was much higher than the stem sequence, in the C-MYC probe the is melting temperature of the loop is 47.6°C and the melting temperature of the stem is 14.1°C. This means that the binding strength of the targeting loop to target mRNA higher that the binding of the stem, due to the loop being longer and containing a higher G-C content, and it allows the stem sequence binding to dissociate upon target DNA binding.

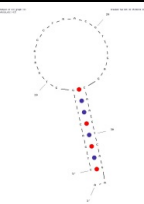
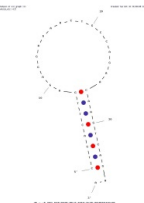
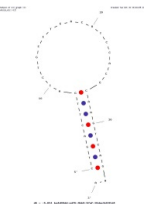
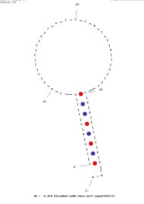
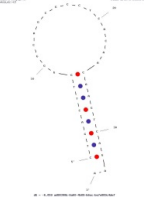
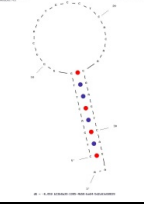
Biomarker	Probe sequence 5' – 3'	Probe length (bp)	Loop length (bp)	Probe ΔG	Probe Tm°C	Stem Tm°C	Loop Tm°C	Probe structure
c-myc	CTGACTTG <u>GTGAAGC</u> <u>TAACGTTG</u> <u>AG</u> CAAGTCA GAA	35	17	-6.01	55.7	14.1	47.6	
Her-1	CTGACTTG <u>AGAGGAG</u> <u>AACTGCC</u> <u>AGAA</u> CAAGTCA GAA	36	18	-5.94	54.9	14.1	52.7	
Her-2	CTGACTTG <u>ATCTGCCT</u> <u>GACATCC</u> <u>ACG</u> CAAGTCA GAA	36	18	-5.65	54.6	14.1	54.7	
Sox-2	CTGACTTG <u>CAGCTCC</u> <u>GTCTCCAT</u> <u>CATGTT</u> CAAGTCA GAA	39	21	-5.36	53	14.1	57	
P53 wildtype	CTGACTTG <u>GGGCAGC</u> <u>GCCTCACA</u> <u>A</u> CAAGTCA GAA	34	16	-6.06	56.6	14.1	59.2	
P53 R175H mutation	CTGACTTG <u>GGGCAGT</u> <u>GCCTCACA</u> <u>A</u> CAAGTCA GAA	34	16	-6.06	56.6	14.1	55.5	

Table 3. Nanoprobe design sequences, melting temperatures and structure for each mRNA biomarker.

Nanoprobes were designed to target multiple different mRNA biomarkers of interest. The nanoprobe sequence was used to determine the melting temperatures and structure. Underlined probe sequences depict the targeting loop position.

5.3.2. Validation of new nanoprobe designs by fluorescence spectroscopy and flow cytometry

Novel nanoprobe designs were designed to target mRNA biomarkers SOX2, HER1 and HER2. Nanoprobes were synthesised using the methods described in Chapter 3 and steady state fluorescence spectroscopy was performed to demonstrate nanoprobe hpDNA opening in response to incubation with cDNA. Each nanoprobe was incubated in 10 mM sodium phosphate buffer pH7 and measured before and after a 2-hour incubation at 37°C with cDNA (Figure 29). Measurements were repeated five times and the average fluorescence emission intensity graphs are plotted.

Each nanoprobe displayed a significant increase in fluorescence after cDNA incubation. SOX2 nanoprobe displayed a 2.1-fold increase in peak fluorescence emission, from 6831.92 (st dev = 673.48) with nanoprobe alone to 15,660.39 (st dev = 1340.67) after cDNA incubation. HER1 nanoprobe displayed a 1.6-fold increase in peak fluorescence emission, from 6674.41 (st dev= 415.35) to 11446.64 (st dev= 535.59). HER2 nanoprobe displayed a 4.4-fold increase in fluorescence emission intensity, from 2926.91 (st dev= 435.39) to 12843.19 (1212.97). The increase in fluorescence of each nanoprobe after cDNA incubation suggests the new nanoprobe designs are successful at recognising target mRNA.

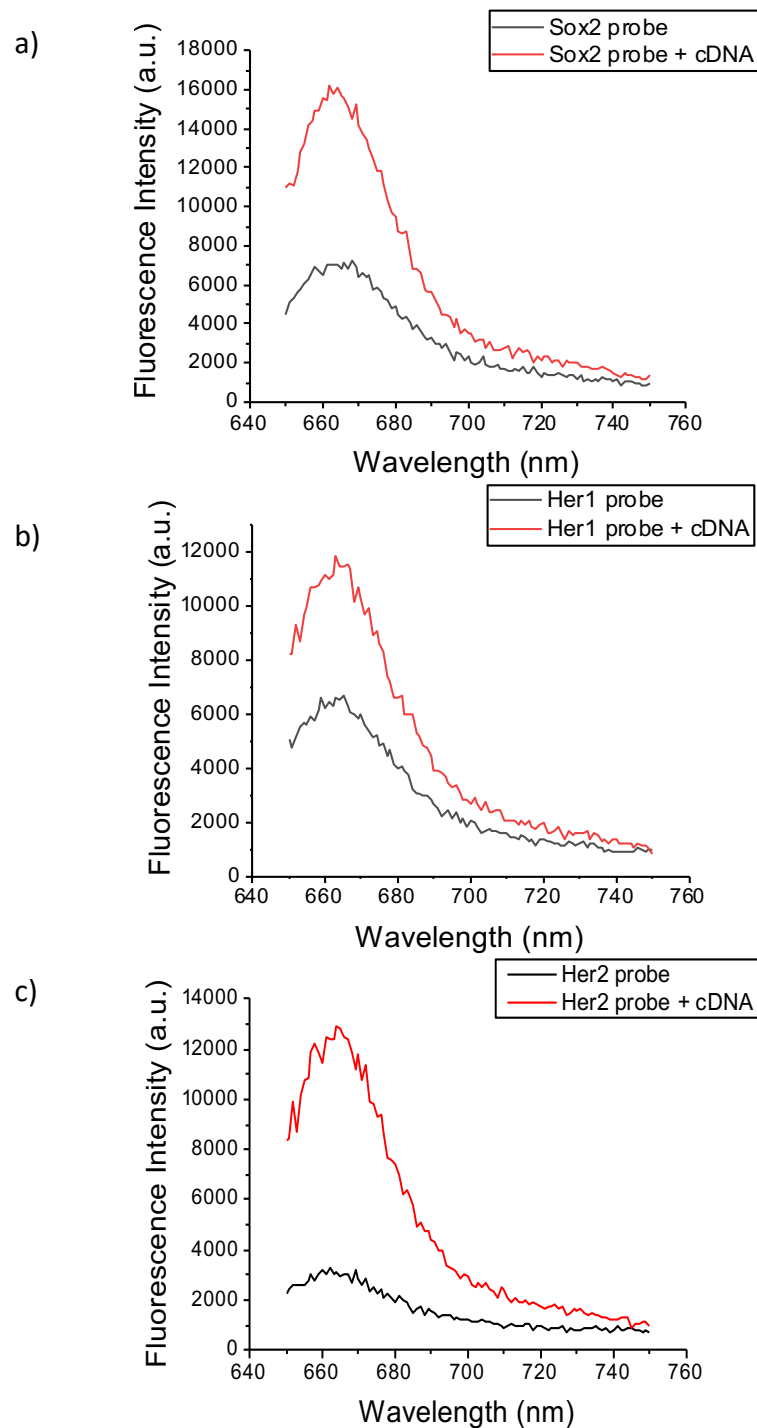


Figure 29. Fluorescence spectroscopy of nanoprobes before and after target DNA incubation.

Nanoprobes designed to target a) SOX2 b) HER1 and c) HER2 mRNA were measured before (black) and after (red) incubation with target DNA. Each nanoprobe displayed different levels of fluorescence background and all exhibited an increase in fluorescence after cDNA incubation.

However, each nanoprobe displayed different initial background fluorescence. This could be due to the differences in nanoprobe design, as different targeting loop sequence may improve or reduce the DNA stability. From Table 3, it can be seen that HER1 and HER2 probes have the same DNA sequence length of 36 nucleotides, while the SOX2 probe has a longer DNA sequence, of 39 nucleotides. Therefore, SOX2 hpDNA should have the highest thermal stability. The HER2 nanoprobe provided the lowest background signal and the highest fluorescence intensity change following cDNA incubation. The background fluorescence observed in HER1 and SOX2 nanoprobe could be due to probe washing efficiency, as washing steps are important for removing unbound hpDNA. If unbound hpDNA remains in the nanoprobe sample following washing steps, this would cause a non-specific background signal in the solution phase spectroscopy measurements as the cy5 would not be in close proximity to the GNR surface and would remain unquenched.

Flow cytometry was also performed with each nanoprobe with mRNA biomarker expressing cell lines (Figure 30). HER1 and SOX2 are biomarkers that are overexpressed in lung cancer, therefore the lung cancer cell line, A549, was used and Hek-293 cells were used as a non-cancer control. HER2 is a biomarker that is overexpressed in a subtype of breast cancers. Two breast cancer cell lines were used; MCF7, which has normal expression of HER2 and SKBR3 which overexpress HER2.

Control cells, GNR treated cells and nanoprobe treated cells were prepared as previously described. For each cell line, control and GNR treated displayed very low background fluorescent signals. Both SOX2 and HER1 lung cancer biomarkers displayed a low fluorescence in non-cancer cells, with SOX2 displaying a fluorescence intensity of 2.67 and HER1 16.43. In A549 lung cancer cell lines, both nanoprobe displayed an increase in fluorescence intensity, with SOX2 displaying 33.6 and HER1 71.67. This resulted in a 12.6 fold increase in fluorescence for the SOX2 nanoprobe and a 4.4 fold increase in the HER1 nanoprobe within lung cancer cells (Figure 30 a,b). The HER1 nanoprobe displayed higher background

fluorescence in the non-cancer cell lines as compared to the SOX2 nanoprobe. Similarly to the fluorescence spectroscopy results, the SOX2 nanoprobe displayed an overall higher fluorescence intensity response. This could suggest that the SOX2 nanoprobe design is more stable, with a lower background fluorescence and a higher binding efficiency to target to mRNA, as it displays the highest fluorescence intensity response within lung cancer cells. However, the high response of SOX2 nanoprobe could also be due to a high expression of SOX2 mRNA within A549 cells.

To determine the potential of the HER2 nanoprobe to distinguish this subtype of breast cancer, two breast cancer cell lines were used. The breast cancer cell line MCF7 was used, which exhibit normal expression of HER2 (173), and compared with the SKBR3 breast cancer cell line, which exhibit an overexpression of HER2 (174). From flow cytometry analysis, the HER2 nanoprobe in MCF7 cells displayed a fluorescence intensity of 10.13 and the same nanoprobe in SKBR3 cells displayed a fluorescence intensity of 21.22. This demonstrates that the HER2 nanoprobe exhibits a 2.2-fold increase in fluorescence in HER2 overexpressing cells, SKBR3, as compared to MCF7 cells (Figure 30c). This provides further evidence that nanoprobe are capable of discriminating cells that overexpress an mRNA biomarker of interest from cells that exhibit normal expression.

Therefore, the potential to design stable nanoprobe against different cancer biomarkers has been demonstrated and the ability of each nanoprobe sequence to detect mRNA biomarkers has been demonstrated in appropriate cancer cell lines.

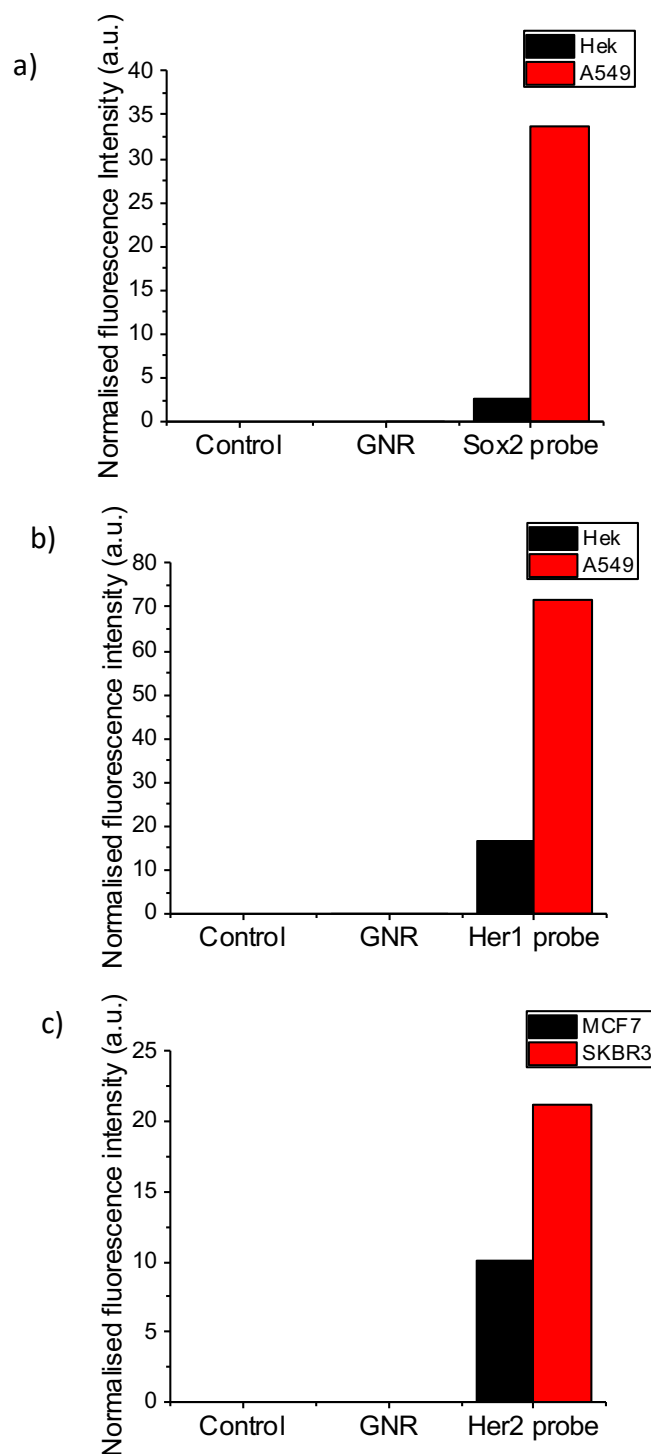


Figure 30. Nanoprobes exhibit fluorescence intensity in target mRNA expressing cell lines.

Nanoprobes were synthesised to target lung cancer biomarkers a) SOX2, b) HER1 mRNA and a breast cancer biomarker c) HER2 mRNA. SOX2 and HER1 nanoprobes were incubated in a non-cancer cell line, Hek-293 (black) and a lung cancer cell line, A549 (red). HER2 nanoprobes were incubated in a breast cancer cell line with normal HER2 expression, MCF7, and a breast cancer cell line that overexpresses HER2, SKBR3. Each nanoprobe displayed a fluorescence signal that was dependent on the expression of the target mRNA biomarker.

5.3.3. Designing nanoprobe against a P53 mutation biomarker

P53 has been discussed as a major genetic player in the development of many different cancer types. Unlike the previous cancer biomarkers that exhibit their tumorigenic response through an overexpression of the gene, P53 promotes tumour growth through mutations that alter normal P53 functions. One of the most common and most studied mutations is the mutation R175H. This mutation results in the change from the amino acid arginine (R), at amino acid position 175, to the amino acid histidine (H). This change is a result of a mutation in the DNA sequence within the genome and it results in an allele change within the RNA sequence from a CGC (wildtype) to a CAC (mutation), where the nucleotide guanine is mutated to an adenine. This single nucleotide change alters the behaviour of the P53 protein and removes the anti-tumour functions that the wildtype protein exhibits. The ability of the nanoprobe to discriminate a one-nucleotide mismatch would be valuable for mutation biomarker detection. As well as investigating the ability of the nanoprobe to detect a mutational biomarker, the effect of the positioning of the mutation within the hpDNA design was also investigated.

Nanoprobes were designed to either target the wildtype sequence of P53 or the R175H mutation sequence of P53. This was designed as a 16 base pair targeting loop, and the mutation targeting sequence was positioned in the middle of the hpDNA targeting loop (design 1), or at the end of the targeting loop (design 2). The structure of each nanoprobe was created using Integrated DNA Technologies Oligo Analyser software, and depicts nanoprobe design against wildtype P53 design 1 (Figure 31a), mutant P53 design 1 (Figure 31b), wildtype P53 design 2 (Figure 31c) and mutant P53 design 2 (Figure 31d). The red circles within each figure depict the positioning of the wildtype or mutation nucleotide sequence.

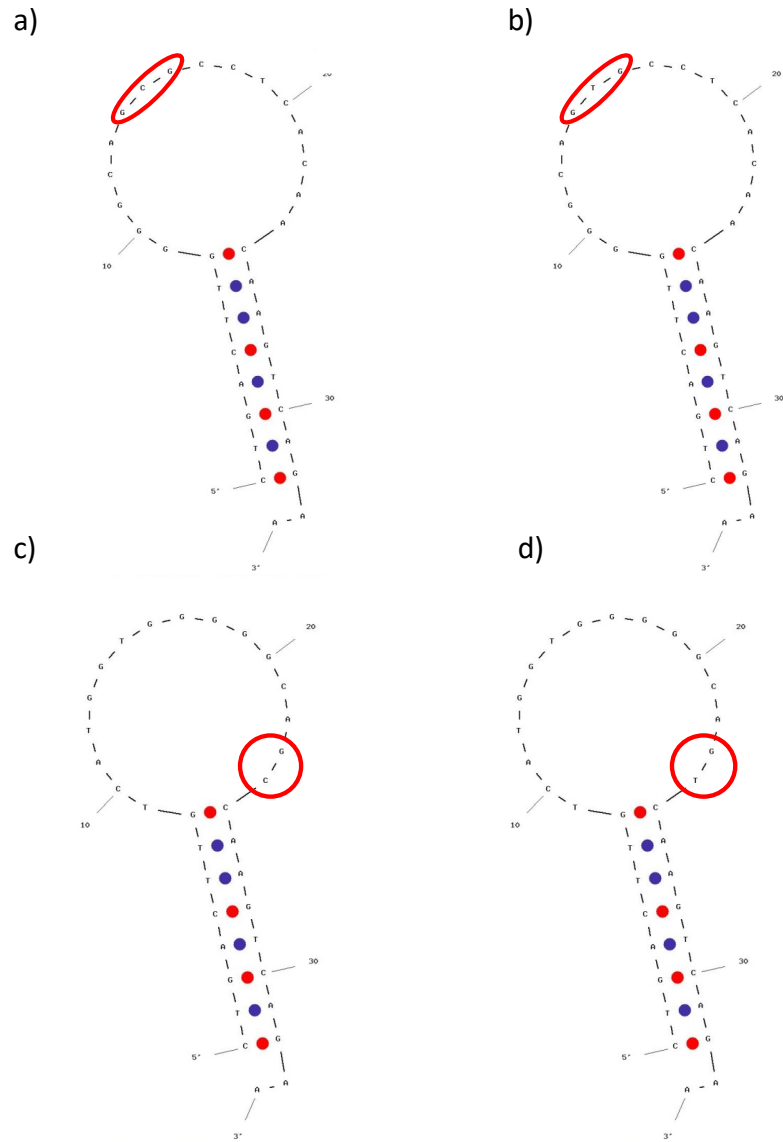


Figure 31. P53 wildtype and mutant nanoprobe designs.

P53 nanoprobe designs were created against wildtype and R175H mutant P53 with two different positionings of the mutation. Design 1 contains the sequence in the middle of the hpDNA targeting loop to bind to a) wildtype P53 or b) mutant P53. Design 2 contains the nucleotide sequence at the end of the hpDNA targeting loop to bind to c) wildtype P53 or d) mutant P53.

Steady state fluorescence spectroscopy was utilised to investigate the nanoprobe opening dynamics in response to a perfect DNA target or a one nucleotide mismatch DNA target. Each nanoprobe was measured alone, to determine the background fluorescence, before being incubated with a perfect cDNA or a single mismatch DNA sequence. Each sample condition was measured five times and the average fluorescence emission intensity was plotted. To determine the relative change in fluorescence, the fold-change for average peak fluorescence emission from nanoprobe with a perfect target was set to 1 and the fold-change with nanoprobe with a single mismatch DNA was compared.

The results for design 1 of the P53 nanoprobe are shown in Figure 32. For the wildtype nanoprobe design 1, the nanoprobe demonstrated a higher fluorescence intensity when incubated with a perfect cDNA target, with an average peak fluorescence change from 4896.85 (st dev= 243.59) to 9501.56 (st dev= 848.87) as compared to incubation with a one nucleotide mismatch, which displayed an increase from 5288.01 (st dev= 836.21) to 9367.85 (st dev= 1497.25) (Figure 32a&b). This shows that with a perfect DNA target, the nanoprobe displayed a 1.82-fold increase in fluorescence, as compared with a single mismatch DNA, which displayed a 1.50 increase in fluorescence.

For the mutant nanoprobe design 1, the nanoprobe demonstrated a higher fluorescence intensity when incubated with a perfect DNA target, with an average peak fluorescence change from 4496.12 (st dev= 152.49) to 7730.93 (st dev= 613.75) as compared to incubation with a one nucleotide mismatch, which displayed an increase from 4415.41 (st dev= 234.73) to 5846.23 (st dev= 255.90) (Figure 32c&d). This shows that with a perfect DNA target, the nanoprobe displayed a 1.72-fold increase in fluorescence, as compared with a single mismatch DNA, which displayed a 1.32 increase in fluorescence.

The relative intensity for each nanoprobe incubation condition was calculated, by comparing the peak fluorescence emission values for each nanoprobe, at 665 nm. For the P53 wildtype probe, the fluorescence intensity after incubation with

wildtype DNA was set to 1.0 (st dev= 0.09). When the wildtype nanoprobe was incubated with mutant P53 DNA, the fluorescence intensity change decreased to 0.82 (st dev= 0.16) (Figure 32e). Similarly for the P53 mutation nanoprobe, the fluorescence intensity following mutant DNA incubation was set to 1.0 (st dev= 0.08) and this reduced to 0.77 (st dev= 0.04) following incubation with wildtype DNA (Figure 32f).

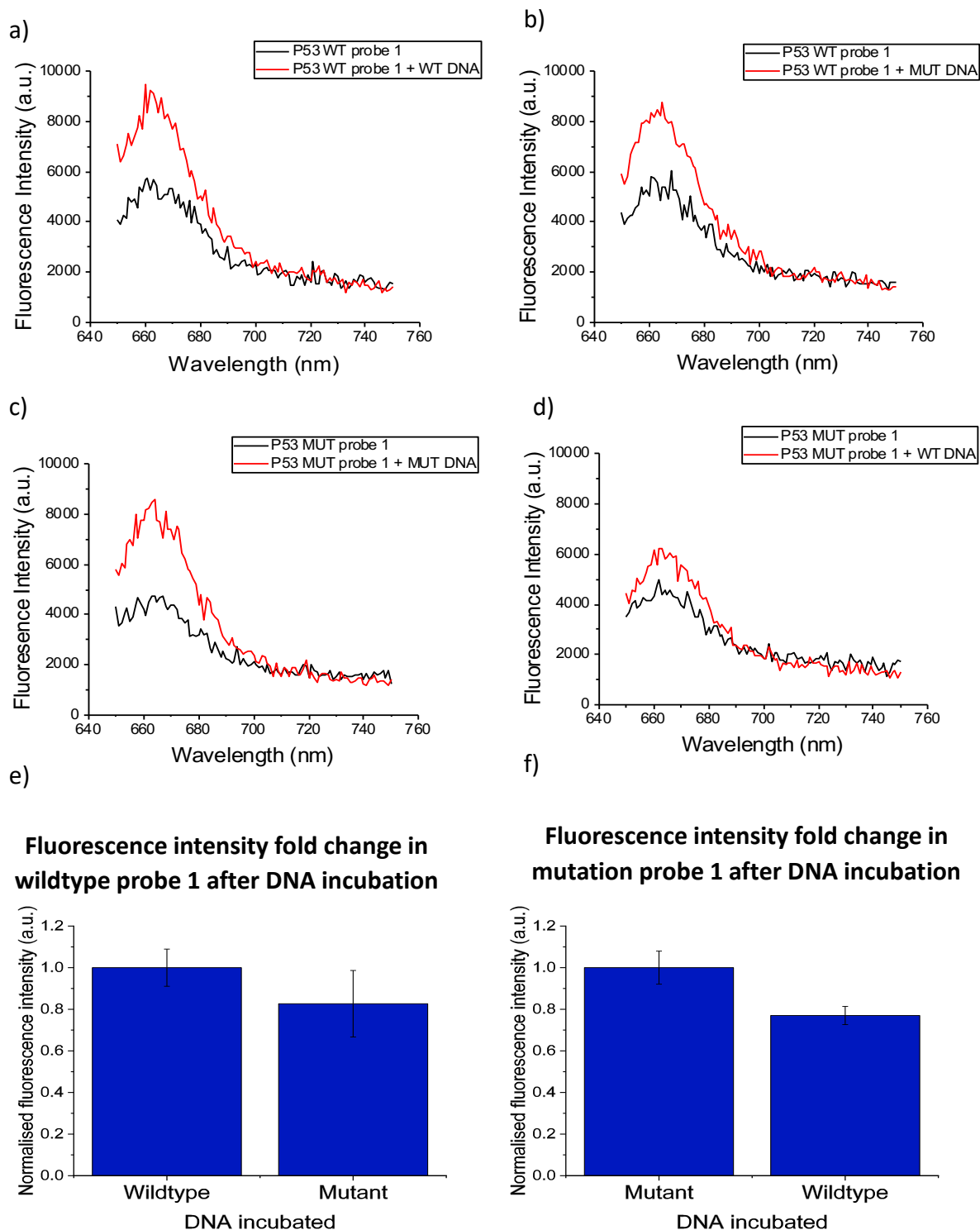


Figure 32. Fluorescence intensity of P53 wildtype and P53 mutation design 1 nanoprobe after complementary or one nucleotide mismatch DNA incubation.

Fluorescence emission spectroscopy measurements of a) wildtype P53 probe with wildtype DNA, b) wildtype P53 probe with mutation DNA, c) mutation P53 probe with mutation DNA and d) mutation P53 probe with wildtype DNA. The fold change in fluorescence emission intensity after complementary or one nucleotide mismatch DNA for e) wildtype P53 probe and f) mutation P53 probe.

The same fluorescence emission measurements were performed with nanoprobe design 2 for wildtype and mutation P53 nanoprobe (Figure 33). For the wildtype P53 nanoprobe design 2, the nanoprobe demonstrated a higher fluorescence intensity when incubated with a perfect cDNA target, with an average peak fluorescence change from 4310.15 (st dev= 702.08) to 9307.15 (st dev= 876.73) as compared to incubation with a one nucleotide mismatch, which displayed an increase from 4465.62 (st dev= 640.87) to 8455.37 (st dev= 251.90) (Figure 33a&b). This shows that with a perfect DNA target, the nanoprobe displayed a 2.52-fold increase in fluorescence, as compared with a single mismatch DNA, which displayed a 1.76-fold increase in fluorescence.

For the mutant P53 nanoprobe design 2, the nanoprobe demonstrated a higher fluorescence intensity when incubated with a perfect cDNA target, with an average peak fluorescence change from 4515.22 (st dev= 589.71) to 9171.28 (st dev= 131.63) as compared to incubation with a one nucleotide mismatch, which displayed an increase from 5441.26 (st dev= 306.44) to 8344.96 (st dev= 258.32) (Figure 33c&d). This shows that with a perfect DNA target, the nanoprobe displayed a 2.03-fold increase in fluorescence, as compared with a single mismatch DNA, which displayed a 1.53-fold increase in fluorescence.

The relative intensity for each nanoprobe incubation condition was calculated, by comparing the peak fluorescence emission values for each nanoprobe, at 665 nm. For the P53 wildtype probe, the fluorescence intensity after incubation with wildtype DNA was set to 1.0 (st dev= 0.09). When the wildtype nanoprobe was incubated with mutant P53 DNA, the fluorescence intensity change decreased to 0.69 (st dev= 0.03) (Figure 33e). Similarly for the P53 mutation nanoprobe, the fluorescence intensity following mutant DNA incubation was set to 1.0 (st dev= 0.01) and this reduced to 0.75 (st dev= 0.03) following incubation with wildtype DNA (Figure 33f).

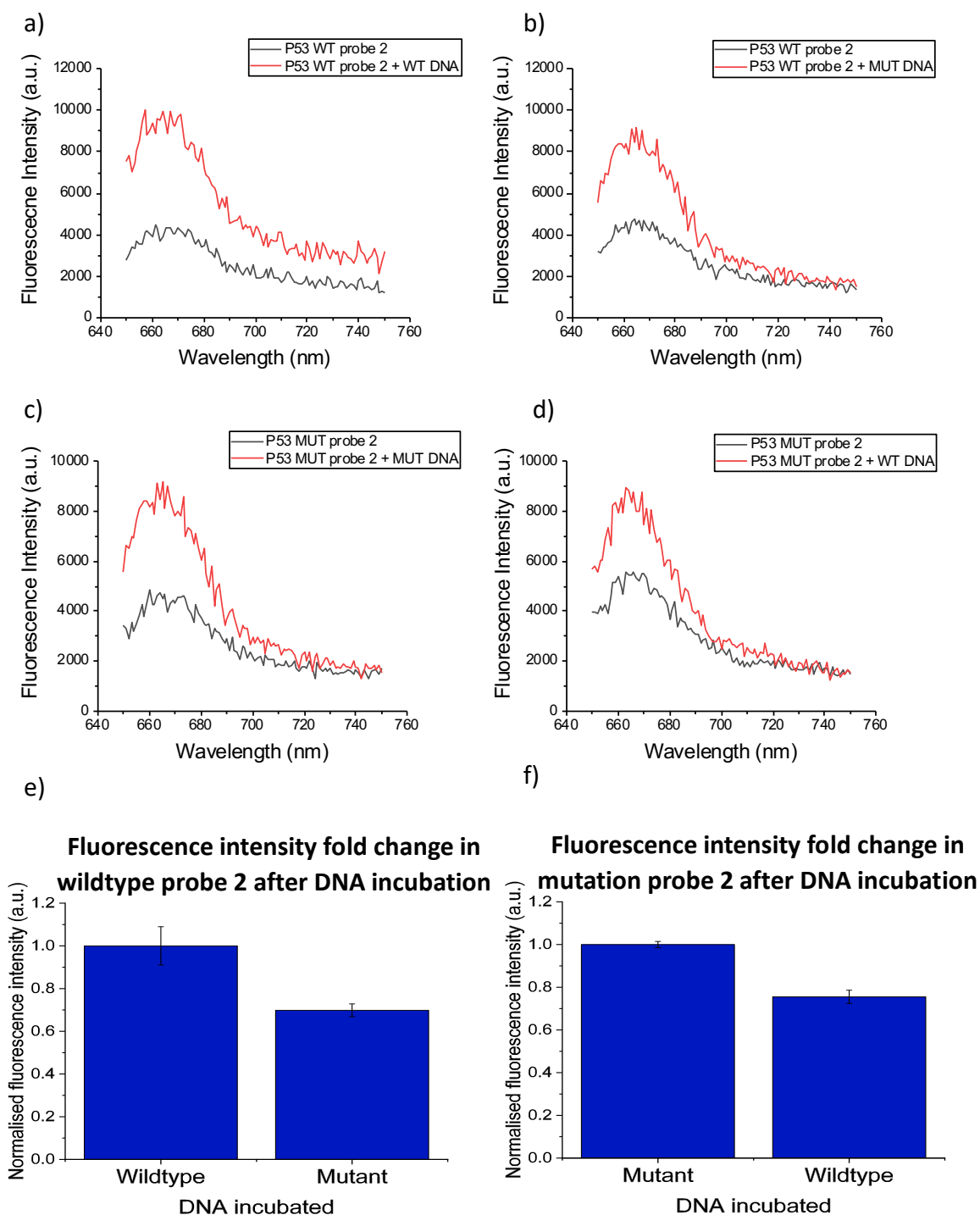


Figure 33. Fluorescence intensity of P53 wildtype and P53 mutation design 2 nanoprobe after complementary or one nucleotide mismatch DNA incubation.

Fluorescence emission spectroscopy measurements of a) wildtype P53 probe with wildtype DNA, b) wildtype P53 probe with mutation DNA, c) mutation P53 probe with mutation DNA and d) mutation P53 probe with wildtype DNA. The fold change in fluorescence emission intensity after complementary or one nucleotide mismatch DNA for e) wildtype P53 probe and f) mutation P53 probe.

These steady state fluorescence spectroscopy measurements in solution phase have demonstrated that the nanoprobe opening dynamics are affected by incubation with a DNA target that contains a one-nucleotide mismatch. This suggests that nanoprobe are sensitive to discriminate a one-nucleotide mutation.

Flow cytometry was performed in cell lines to determine the sensitivity of P53 nanoprobe in differentiating mRNA targets with a one-nucleotide mismatch (Figure 34). Both design 1 and design 2 of wildtype and mutant P53 nanoprobe were incubated with Hek cells, as a non-cancer control that exhibits low expression of wildtype P53. Nanoprobe were also incubated in Flo-1 cells, which are an oesophageal cancer cell line that exhibit an overexpression of wildtype P53. Therefore, the ability of the mutation nanoprobe to discriminate wildtype target expression was investigated.

Both cell lines were left untreated as a control or incubated with 0.1 nM of GNRs or nanoprobe for 6 hours. Cells were collected and the fluorescence intensity was measured by flow cytometry. Control cells and GNR treated cells displayed very low fluorescent signals. In nanoprobe design 1, where the mutation sequence is positioned in the middle of the hpDNA targeting loop, the fluorescence intensity in Hek cells following incubation with wildtype P53 was 2.54 and following incubation with mutant P53 was 2.20. Therefore, the fluorescence intensity was low and similar in Hek cells after incubation with both P53 wildtype or mutant nanoprobe. In Flo-1 cells, which express wildtype P53, following incubation with P53 nanoprobe design 1, the fluorescence signal from the P53 wildtype nanoprobe (17.74) was more than double the signal from the P53 mutant nanoprobe (7.72) (Figure 34a).

In nanoprobe design 2, where the mutation sequence is positioned near the end of the hpDNA targeting loop, the wildtype P53 nanoprobe again displayed low fluorescence intensity in Hek cells, with an intensity of 7.7 and an intensity of 5.5 following incubation with mutation P53 nanoprobe. Following incubation with Flo-1 cells, a higher fluorescence intensity was observed with the P53 wildtype nanoprobe of 30.2, as compared to the P53 mutant nanoprobe which displayed a

fluorescence intensity of 26.9. However, the difference in fluorescence intensity was not as pronounced as the nanoprobe design 1 (Figure 34b).

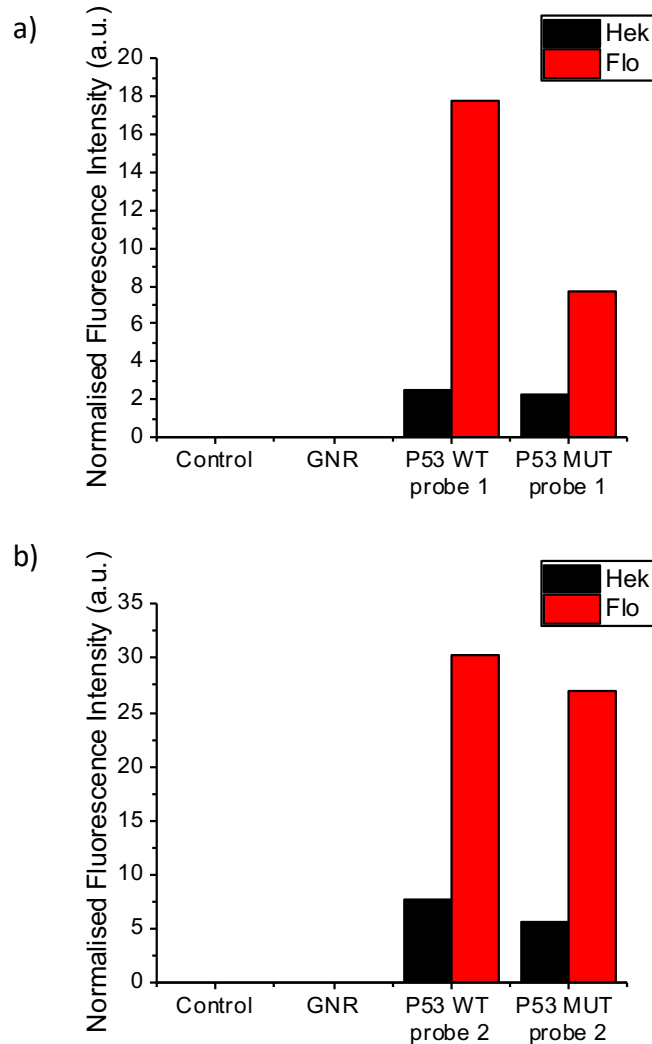


Figure 34. Flow cytometry of wildtype and mutant P53 nanoprobes in P53 wildtype expressing cells demonstrates differential fluorescence.

Wildtype and P53 nanoprobes were incubated in low P53 expressing Hek cells and wildtype P53 expressing Flo cells. Two nanoprobe designs were compared, a) design 1 contains the P53 mutation in the middle of the hpDNA loop and b) design 2 which contains the mutation sequence at the end of the hpDNA loop. In both designs, the mutant nanoprobe displayed a reduced fluorescence intensity as compared with the wildtype nanoprobe. This suggests that nanoprobe binding and opening is reduced when there is one nucleotide mismatch.

To determine the difference in binding efficiency between nanoprobe designs, the binding selectivity coefficient was calculated for each nanoprobe. The binding selectivity coefficient, α , can be calculated by comparing binding to a perfect target and a one nucleotide mismatch target (175) and can be defined as:

$$\alpha = (S/B)_{i,j} / (S/B)_{i,j'}$$

Equation 7.

Where $(S/B)_{i,j}$ is the signal to background ratio for the nanoprobe (i) in the presence of a DNA target (j), and $(S/B)_{i,j'}$ is the signal to background ratio for the nanoprobe (i) in the presence of a DNA target (j'). The S/B was defined as:

$$(F_{\text{hybrid}} - F_{\text{buffer}}) / (F_{\text{probe}} - F_{\text{buffer}})$$

Equation 8.

Where F_{buffer} , F_{probe} , and F_{hybrid} are the fluorescence intensities of the buffer solution, the nanoprobe without target, and the nanoprobe after target incubation, respectively. For flow cytometry calculations, F_{buffer} , F_{probe} , and F_{hybrid} were calculated from the fluorescence intensities of Flo-1 cells alone, the nanoprobe in Hek cells and the nanoprobe in Flo-1 cells, respectively.

The selectivity of the nanoprobe with a complementary target was used as a reference ($\alpha=1$). From this, the selectivity coefficient of nanoprobe binding to mismatch DNA targets can be calculated. The selectivity coefficient for each P53 nanoprobe with a single mismatch DNA target was calculated for solution phase fluorescence emission measurements (Table 4 & Table 5) and for flow cytometry measurements in cells (Table 6).

P53 probe design 1 solution phase	
Signal to background ratio	
WT probe 1 + WT DNA	2.45
WT probe 1 + MUT DNA	2.08
MUT probe 1 + MUT DNA	2.16
MUT probe 1 + WT cDNA	1.50
Selectivity coefficient	
WT probe 1 + WT DNA	1.00
WT probe 1 + MUT DNA	0.85
MUT probe 1 + MUT DNA	1.00
MUT probe 1 + WT cDNA	0.69

Table 4. The selectivity coefficient of P53 nanoprobes design 1 in solution phase.

P53 probe design 2 solution phase	
Signal to background ratio	
WT probe 2 + WT DNA	3.70
WT probe 2 + MUT DNA	2.19
MUT probe 2 + MUT DNA	2.66
MUT probe 2 + WT DNA	1.78
Selectivity coefficient	
WT probe 2 + WT DNA	1.00
WT probe 2 + MUT DNA	0.59
MUT probe 2 + MUT DNA	1.00
MUT probe 2 + WT DNA	0.67

Table 5. The selectivity coefficient of P53 nanoprobes design 2 in solution phase.

P53 nanoprobe flow cytometry	
Signal to background ratio	
WT probe 1 + WT cells	6.96
MUT probe 1 + WT cells	2.97
WT probe 2 + WT cells	3.84
MUT probe 2 + WT cells	3.39
Selectivity coefficient	
WT probe 1 + WT cells	1.00
MUT probe 1 + WT cells	0.43
WT probe 2 + WT cells	1.00
MUT probe 2 + WT cells	0.88

Table 6. The selectivity coefficient of P53 nanoprobe in Flo-1 cells by flow cytometry.

These calculations show that in flow cytometry, the signal to background ratio is much higher as compared with solution phase fluorescence emission measurements. This could be due to unbound fluorophore labelled hpDNA present in the nanoprobe in solution phase after the washing steps. However, in flow cytometry this unbound hpDNA would not be uptaken by cells and would be washed away, therefore the fluorescence signal is only from nanoprobe that enter cells and bind to target mRNA.

In steady state fluorescence spectroscopy in solution phase, the binding efficiencies of each mismatch DNA incubation was reduced, as compared to incubation with perfect complementary DNA. For P53 nanoprobe design 1, the selectivity coefficient was 0.85 for the wildtype probe incubated with mutant DNA and 0.69 for the mutant probe binding to wildtype DNA. For P53 nanoprobe design 2, the selectivity coefficient was 0.59 for wildtype probe incubated with mutant DNA, and 0.67 for mutation probe incubated with wildtype DNA. Therefore, both nanoprobe exhibit reduced binding affinity to a sequence with a one-nucleotide mismatch. However,

the positioning of the mismatch did not result in a clear difference in the binding affinity.

For flow cytometry measurements, both wildtype and mutation P53 nanoprobe were incubated in P53 wildtype expressing cells, Flo-1. The binding efficiency of the P53 mutant nanoprobe was reduced to 0.43 when one nucleotide mismatch is present and when the mismatch is located in the centre of the probe design (design 1). When the mismatch is located at the side of the nanoprobe design (design 2), the binding efficacy was reduced to 88%. Therefore, both mutation nanoprobe designs showed a reduced binding affinity to wildtype P53, when a one-nucleotide mismatch was introduced, but the nanoprobe where the mutation was located in the centre of the nanoprobe hpDNA loop was much more effective at discriminating wildtype P53 from mutation P53.

A similar study using molecular beacons showed that a FAM labelled MB detected a single mismatch mutation with a selectivity coefficient of 0.73 and the same MB labelled with cy5 showed a selective coefficient of 0.78 (175). The single mismatch in this sequence was located in the centre of the DNA recognition sequence, which is comparable to the P53 mutation nanoprobe design 1. Therefore, the nanoprobe provides higher binding selectivity to single mismatch sequences compared to reported MB designs.

The response of nanoflares to a target with one nucleotide mismatch have previously been investigated and this showed that nanoflare fluorescence decreased by 48% in response to a single mismatch mRNA as compared to a perfect complimentary target (176). The nanoprobe showed a reduction of 57% in response to a one nucleotide mismatch located in the centre of the targeting loop. Therefore, nanoprobe offer an enhanced ability to discriminate target mRNA biomarker mutations.

Unfortunately, a cell line that expresses the R175H P53 mutation was unable to be acquired. This would have allowed the same experiment to be performed to investigate if there was differential fluorescence from the wildtype P53 nanoprobe

in cells that expressed the mutant biomarker. It would be expected that a similar reduction in the selectivity coefficient would be found for wildtype P53 nanoprobe in cells that express the mutation mRNA.

5.4. Conclusions

In this investigation, nanoprobe have been successfully designed and developed in fluorescence spectroscopy and flow cytometry for the detection of several cancer mRNA biomarkers. This investigation was significant as it shows that nanoprobe designs can be easily created and validated against multiple different mRNA biomarkers of interest. The high fluorescence intensity of the nanoprobe within cancer cells that overexpress the mRNA biomarker as compared to the low fluorescence intensity in cells with low or normal biomarker mRNA expression demonstrates the specificity of the nanoprobe to their target and the stability of nanoprobe not opening randomly within cells.

Following the potential to detect multiple different mRNA biomarkers within cells, the ability for nanoprobe to detect mutational mRNA biomarkers was demonstrated. Nanoprobe were designed and synthesised against wildtype P53 and R175H mutation P53 mRNA biomarkers. Nanoprobe targeted against the mutation P53 biomarkers were designed with the mutation at two different positions; one with the mutation nucleotide at the centre of the hpDNA targeting loop and the second with the mutation at the edge of the targeting loop.

P53 wildtype and mutation nanoprobe were investigated by steady state fluorescence spectroscopy and flow cytometry. This demonstrated that when the single mismatch bp mutation was located at the centre of the hpDNA targeting loop, the fluorescence intensity of the nanoprobe in cells expressing P53 with a mismatch reduced by 57%, compared to the nanoprobe in cells with a perfect complimentary P53 mRNA sequence. Furthermore, when the mutation was located at the side of the hpDNA, the fluorescence intensity of the nanoprobe reduced by 12%.

This investigation is the first time that nanoprobe have been shown to be capable of discriminating a one-nucleotide mismatch within the target-binding loop. Furthermore, the positioning of the single nucleotide mismatch within the hpDNA-targeting loop within the nanoprobe design has been shown to be essential in effecting the nanoprobe binding selectivity coefficient. This investigation has demonstrated the potential of nanoprobe, not only for mRNA biomarkers that are overexpressed, but also for the detection of single nucleotide mutation mRNA biomarkers. Therefore, nanoprobe have the capability of differentiating targets of a single mismatch, providing the potential for nanoprobe to be used in clinical liquid biopsies to detect mutational cancer mRNA biomarkers.

6. Demonstrating the internalisation of nanoprobe and target mRNA specific fluorescence in individual cells by confocal fluorescence microscopy & fluorescence lifetime imaging microscopy

6.1. Introduction

The function of nanoprobe for the detection of CTCs is dependent on cellular uptake and binding to target mRNA. Although there have been many studies investigating the process by which nanoparticles enter cells and their intracellular fate, the mechanism which control these processes are still under debate. This investigation is not on the mechanism of nanoparticle uptake, but an investigation to visualise nanoprobe internalisation and fluorescence. However, it is important to have an understanding of uptake mechanisms that may be involved in nanoprobe uptake.

Subcellular compartmentalisation is a highly controlled and dynamic process that plays an important role in cellular biochemical reactions and cell communication. Vesicles, which are small membrane enclosed sacks, control the trafficking of internal materials and they contain materials such as proteins and mRNAs. Materials are continuously being taken into a cell by vesicles, through endocytosis, and moved out of a cell, through exocytosis.

There is growing evidence that nanoparticles enter cells through the endocytosis pathway (177). Based on the size of endosomes, it has been suggested that nanoparticles from 10 - 300 nm could be internalised through the clathrin-mediated endocytosis pathway. Studies have demonstrated that nanoparticles up to 100 nm in diameter can be uptaken through this pathway (178).

Vesicles that are formed from materials entering the cells through endocytosis are called endosomes. Endosomes act as a temporary mode of transport of materials that are taken into cells and can either deliver cargo to a target areas within a cell, fuse with a lysosome for degradation, or return to the cell surface (179). Therefore,

nanoparticles entering cells are at risk for degradation or recycled back outside the cell.

There is also evidence that nanoparticles can escape endosomes and be released into the cytoplasm (Figure 35) (180). A number of methods have been suggested to allow nanoparticle escape. Nanoparticles have been designed to cause osmotic pressure that results in the rupture of endosome membranes (181). Particles have also been designed to swell in the low pH conditions of the endosome, resulting in endosome breakdown (182). There have also been reports that nanoparticle interactions with the endosome membrane can result in the release of the cargo into the cytosol (183).

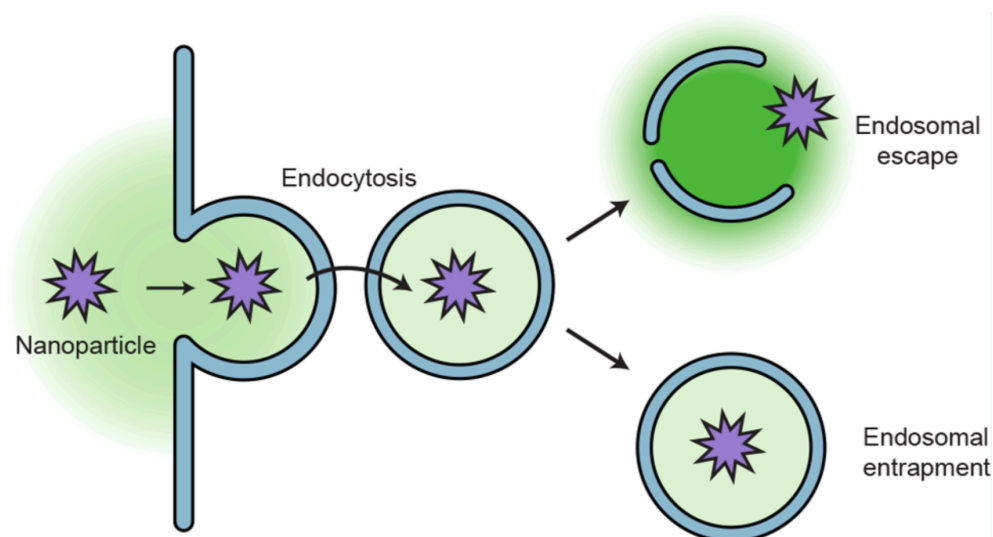


Figure 35. Fate of nanoparticles following endocytosis.

Nanoparticles can be taken into cells through endocytosis, which is the engulfment into endosomes. Once in endosomes, nanoparticles can either escape into the cytosol or be transported to a subcellular compartment or be degraded. This figure was used with permission from ASC publications, Copyright © 2019, American Chemical Society.

Due to the large surface area of nanoparticles it has also been suggested that interface interactions, such as electrostatic and van der Waals forces could modulate nanoparticle interactions and uptake by the cell membrane (184). There is evidence to suggest that interactions between nanoparticles over 30 nm in diameter and the cell membrane can result in a deformation in the membrane, due to the interaction between a solid nanoparticle and the membrane fluidity, that results in the engulfment of these particles (185). Theoretical models have calculated that clusters of nanoparticles enhances this effect, as an increase in the nanoparticle interactions with the membrane lowers the energy required for membrane engulfment (186).

The uptake of nanoparticles and their internal fate is also greatly dependent on the size, charge and composition of the particle. Nanoparticle interactions with the cell membrane have been shown to disturb the integrity of the membrane in a size dependent manner, with studies showing that nanoparticles of 50 nm are the optimal size to achieve highest uptake (187). Simulations have shown that surface charge has a significant role on nanoparticle uptake. Positively charged nanoparticles have been shown to have a disruptive influence on the membrane stability and promotes uptake by membrane wrapping, whereas negatively charged nanoparticles were shown to have a higher penetration efficiency through the membrane (188). This is thought to be due to interactions with the phosphate groups of the lipids within the membrane, which increases the surface tension and results in the formation of pores in the membrane (189).

Furthermore, the shape of nanoparticles also determines the intracellular fate. The downfall of the use of spherical nanoparticles are they are more likely to be trapped in the endosomal pathway than GNRs, where they can be degraded by lysosomes or transported back out of the cell through exocytosis. A study of the internal fate of nanoflares showed through electron microscopy that the particles remained within endocytic compartments and did not report on mRNA levels (190). In comparison, the benefits of using GNR particles are they have been shown to be more likely to escape the exosome pathway. Studies into the effect of nanoparticle shape on their

intracellular trafficking found that the ability of nanoparticles to escape endosomes was dependent on the circularity, with particles with less circularity, i.e. sharper nanoparticles, displaying higher escape compared with circular nanoparticles (191). Furthermore, TEM imaging of nanoparticle incubated breast cancer cells demonstrated that GNRs do escape endosomes (192).

The uptake of fluorescent GNRs functionalised with a targeting oligonucleotide has previously been demonstrated by fluorescence imaging. A study that generated GNRs functionalised to target telomerase mRNA, which is activated in cancer cells but not in healthy cells, was investigating the potential for targeted nanorods to induce apoptosis by inhibiting the target mRNA activity. It was demonstrated through TEM and confocal fluorescence microscopy that within 3 hours of GNR incubation, the GNRs were localised to the cytoplasm of the cell and after 24 hours of incubation the GNRs had entered the nucleus and bound to telomerase mRNA, which induced cell apoptosis (193). This study demonstrated the ability of GNRs to enter cells and bind to their intracellular targets.

The intracellular fate of nanoprobe is critical to the ability to detect target mRNA. Two fluorescence imaging techniques were utilised to provide different but complimentary evidence that nanoprobe are uptaken into cells and display target binding specific fluorescence.

GNRs provide high contrast agents in cellular imaging, due to their high light scattering properties at the visible and near infrared wavelengths. GNRs can also be labelled with fluorescent dyes for imaging applications. Confocal fluorescence microscopy is a powerful and well-established imaging technique and has been used to study gold nanoparticle uptake and distribution within cells.

More specifically, fluorescence microscopy has also been utilised to study RNA biomarkers and RNA dynamics within cells. The translocation of mRNA molecules from the nucleus to the cytoplasm was first visualised by high-resolution fluorescence microscopy (194). As mRNA sensing is of interest for disease targeting and disease diagnostic applications, there has been the development of new probes

and new imaging techniques. Many of these techniques function as a hybridisation probe, for the specific binding to unique mRNA sequences. The sensitivity and resolution of imaging techniques are continuously improving, allowing the spatial distribution of RNAs to be investigated.

Single molecule sensitivity fluorescent in situ hybridization (smFISH) is a technique that has recently been utilised to study RNA expression. This technique relies on the binding of many short oligonucleotide probes to complimentary RNA sequences. The binding of multiple probes to one mRNA molecule amplifies the fluorescence signal, therefore the target signal can be identified over non-specific binding of individual probes (195). However, a limitation of this process is that it is not capable of detecting single nucleotide mutations between RNA sequences.

mRNA fluorescence imaging has also been achieved in live cells (196). mRNA particles were detected through fluorescently labelled oligonucleotides. Using light sheet microscopy, optical sectioning allowed 3D localisation of mRNA molecules more than 100 μ m deep in living tissue. However, for live cell imaging techniques these probes require transfection into cells by microinjection and there has been concern that perforation of the membrane may cause cell death and unreliable results.

As well as scattering and fluorescence imaging, GNRs can undergo surface plasmon enhanced two photon luminescence (TPL). TPL provides multiple advantages to imaging, such as the ability to resolve 3D structures, images at deeper depths than conventional microscopy of up to 1 mm, avoids photobleaching and performs in the near infrared region where background from biological tissue is avoided. GNRs are capable of producing a strong TPL following excitation with a pulsed laser source.

FLIM is an imaging technique where each pixel is defined by the fluorescence lifetimes that are present. FLIM makes use of local environment effects that can induce lifetime changes to a fluorophore, such as FRET. FLIM in cells can provide information on the location, conformation and interactions of a fluorophore within a biological environment. With FLIM, TCSPC measurement is used and this occurs

on each individual pixel within the image. The imaging area undergoes scanning cycles to build a lifetime map across the area of interest.

In contrast to fluorescence intensity imaging, fluorescence lifetime is independent of the local concentration of fluorophores, photobleaching, local excitation light intensity and local fluorescence detection efficiency. Furthermore, the fluorescence decay of fluorophores often depends on their intrinsic characteristics and local environment conditions, such as pH, viscosity and temperature, as well as interactions with other molecules such as collisional quenching or energy transfer. In the case of the nanoprobe, the fluorescence decay time depends on the hybridisation status of the hpDNA, which is directly correlated to the concentration of target mRNA.

Previous studies have utilised FLIM to investigate the fluorescence lifetime changes of fluorophores in close proximity to gold nanoparticles. This found that when a fluorophore was in close proximity to a gold nanoparticles, whose longitudinal surface plasmon resonance band overlapped with the fluorophores emission, the fluorescence lifetime of the fluorophore was greatly reduced, due to energy transfer (197).

The fluorescence lifetime of a fluorophore is dependent on its local environment, including interactions such as FRET. Therefore, fluorescence lifetime imaging can provide information on the binding dynamics of the nanoprobe hpDNA design. Previous results shown in Chapter 3 demonstrated that time resolved fluorescence spectroscopy of the nanoprobe in solution phase, the lifetime of cy5 exists in a quenched state when the hpDNA is closed and fluorescence lifetime recovery is observed after target binding.

FLIM of GNRs provides advantages over conventional fluorescence imaging as it provides contrast between the components of each pixel according to the fluorescence decay time. This provides the capability to identify overlapping fluorescence emission species due to differences in their fluorescence lifetime. Gold nanorods have been shown to provide good contrast agents in cellular imaging, due

to their very short fluorescence lifetime of <100 ps (198,199). FLIM has been used to demonstrate that gold nanoparticles which have a spectral overlap with the emission of a fluorophore and are in close proximity have a strong effect on reducing the fluorescence lifetime of the fluorophore (200). Previous studies have also utilised the FLIM-FRET method to demonstrate that gold nanorods are uptaken through endocytosis and are localised in early endosomes (201). Furthermore, FLIM has been shown to be capable of detecting differences between quenched and unquenched cy5 in cells treated with nanoflares (202). Therefore, two-photon lifetime imaging of nanoprobe can provide information on GNR cellular location, as well as the lifetime components and binding state of the nanoprobe.

This imaging study has investigated the fluorescence intensity and lifetimes of C-MYC nanoprobe in cells with low C-MYC mRNA expression (Hek), in comparison with C-MYC overexpressing cancer cells (PC3). Using two imaging techniques, the mRNA expression within cells has been shown to correlate to the fluorescence intensity of the nanoprobe and the fluorescence lifetime. Fluorescence confocal microscopy has demonstrated differentiation of cell lines with different expression levels of target mRNA. Using FLIM, a fast bi-exponential fluorescence lifetime analysis method was employed to provide location mappings of target mRNA in cells. Fluorescence imaging of nanoprobe provides a new way for sensing RNA biomarkers at the single cell level.

6.2. Sample preparation

6.2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) (D5796), non-essential amino acid solution (M7145), sodium pyruvate (S8636), penicillin-streptomycin (P4333) fluoroshield with DAPI (F6057), formaldehyde 4% solution (1004968700), poly-L-lysine solution (P4707) and Eukitt quick-hardening mounting medium (03989) were purchased from Sigma- Aldrich. Fetal bovine serum (FBS) (11550356) and trypsin TrypLE express enzyme (12604013) were purchased from Fisher Scientific. Flow Check Pro Fluorospheres (A63493) was purchased from Beckman Coulter.

6.2.2. Preparing cells on coverslips

Sterile 13 mm coverslips were placed in a 24-well plate and coated with 200 μ l poly lysine (Sigma) for 20 minutes. Poly lysine was removed, and coverslips were washed with distilled water 3 times. Coverslips were left to dry overnight. 100,000 cells were seeded to each coverslip with 0.5 ml media per well and left overnight to seed. The following morning, 0.1 nM of GNRs or nanoprobe were added to each well and incubated for 6 hours. Following incubation, the media was removed, and cells were washed 3 times with PBS. Cells were fixed with 4% paraformaldehyde for 15 minutes. This was then removed, and the cells were washed three times with distilled water. Glass slides were prepared, and a dot of mounting media was added to each slide. For confocal fluorescence imaging, Fluoroshield Mounting Medium with DAPI was used, while for FLIM Eukitt Quick-hardening mounting medium was used. Coverslips were mounted on the glass slide and left overnight to dry. Each coverslip sample was prepared in duplicate.

6.2.3. Confocal imaging

Fluorescence microscopy was carried out on a confocal microscope (Leica Microsystems, SP8 + picoEMERALD S + CW vis lasers) using LASX software. The dye assist software was used to sequentially image DAPI and cy5. DAPI was imaged with 405 nm excitation and emission was collected between 410 – 585 nm with a PMT detector. Cy5 was imaged with 633 nm excitation and the emission was collected between 638 – 779 nm with a HyD detector. Each image was taken with a 60x water-immersion objective with a line average of 4 and a resolution of 1024 x 1024.

Z-stacks images were set up through the Z-position tab. The z position was manually adjusted to the top and bottom extremities of the cell of interest. Both DAPI and cy5 were imaged in separate channels and the final z-stack image was merged.

6.2.4. Fluorescence lifetime imaging

Fluorescence lifetime imaging microscopy was performed on a confocal microscope (LSM 510, Carl Zeiss) equipped with a TCSPC module (SPC-830, Becker &

Hickl GmbH). A femtosecond Ti:Sapphire laser (Chameleon, Coherent) was tuned to 800 nm to generate two photon luminescence from both gold nanorods and cy5. Emission was collected by a 60x water-immersion objective with a 700 nm short pass filter. 100 cycles were performed for each measurement to build up a lifetime image.

6.3. Results

6.3.1. Visualisation of nanoprobe uptake in cells using fluorescence confocal microscopy

Flow cytometry has demonstrated the ability to detect the fluorescence of nanoprobes in individual cells. In order to confirm that nanoprobes are uptaken by cell lines, confocal fluorescence microscopy was performed. Hek and PC3 cells were left untreated as a control or incubated with 0.1 nM of GNRs or nanoprobes for 6 hours. Following incubation, cells were fixed and mounted onto slides with DAPI nuclear staining. Cells were imaged for both DAPI (blue), which was excited with 405 nm, and cy5 (red), which was excited at 633 nm.

Figure 36 shows typical single photon fluorescence intensity images of a control, GNR and nanoprobe treated Hek and PC3 cells. Focusing was performed around the nuclear area to take an image inside the cell. From visual analysis, it appears that there is no red fluorescence from control cells. In GNR treated cells, some red signal can be observed which may be from the scattering of GNRs. In nanoprobe treated cells, red dots can be visualised, and in PC3 cells this is associated with a strong red fluorescent signal.

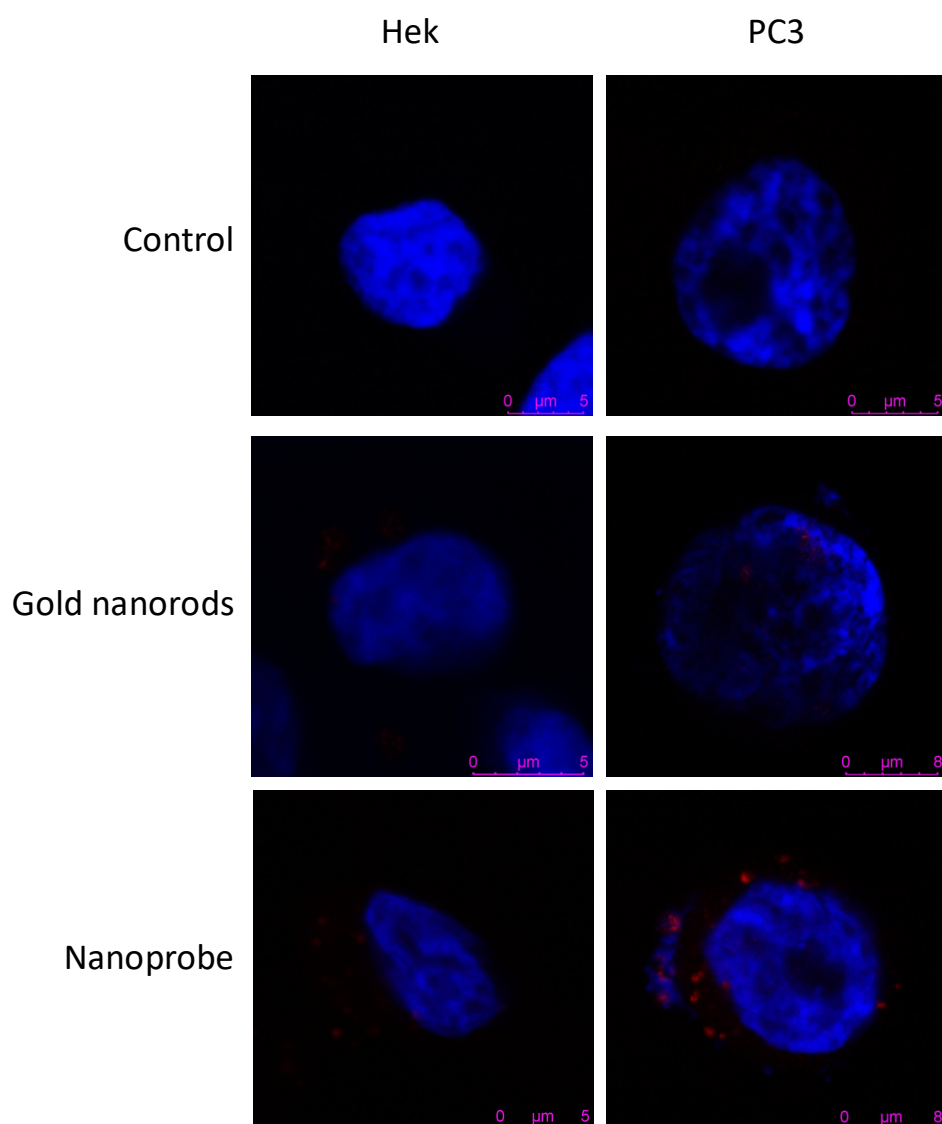


Figure 36. Fluorescence imaging of gold nanorods and nanoprobe in Hek & PC3 cells.

Fluorescence imaging of control, gold nanorod or nanoprobe treated Hek and PC3 cells. The emission of DAPA (blue) and cy5 (red) was collected and the channels overlayed.

Fluorescence analysis was performed on 3 control cells, 3 GNR treated cells and 10 nanoprobe treated cells for each cell type. Analysis was performed in image j. A circle of 28 x 28 pixels was used to measure the mean fluorescence of control cells, GNR spots or nanoprobe spots around each cell. The fluorescence was then

corrected by subtracting the background fluorescence of the image. This analysis was repeated for each image for multiple cell analysis. The fluorescence was analysed by the corrected fluorescence from each spot within each cell (Figure 37a), and as an average collected fluorescence from all spots (Figure 37b).

Individual spot analysis shows that both control Hek and PC3 cells exhibit a very low fluorescence signal. Unlike flow cytometry results, fluorescence imaging analysis indicates that GNR treated cells display a low fluorescence signal, which could be due to scattering of laser light. For nanoprobe treated samples, a total of 93 nanoprobe spots were analysed for Hek cells and a total of 95 nanoprobe spots for PC3 cells. Nanoprobe treated Hek cells displayed a range of fluorescence intensities, ranging from 3 to 18. Nanoprobe treated PC3 cells also displayed a range of fluorescence values, from 8 to 81. When these values were averaged, nanoprobe treated Hek cells display an average fluorescence of 7, while nanoprobe treated PC3 cells display an average fluorescence of 23. Therefore, fluorescence imaging of nanoprobes in target expressing cancer cells display a 3.3 increase in fluorescence, as compared to non-cancer cells.

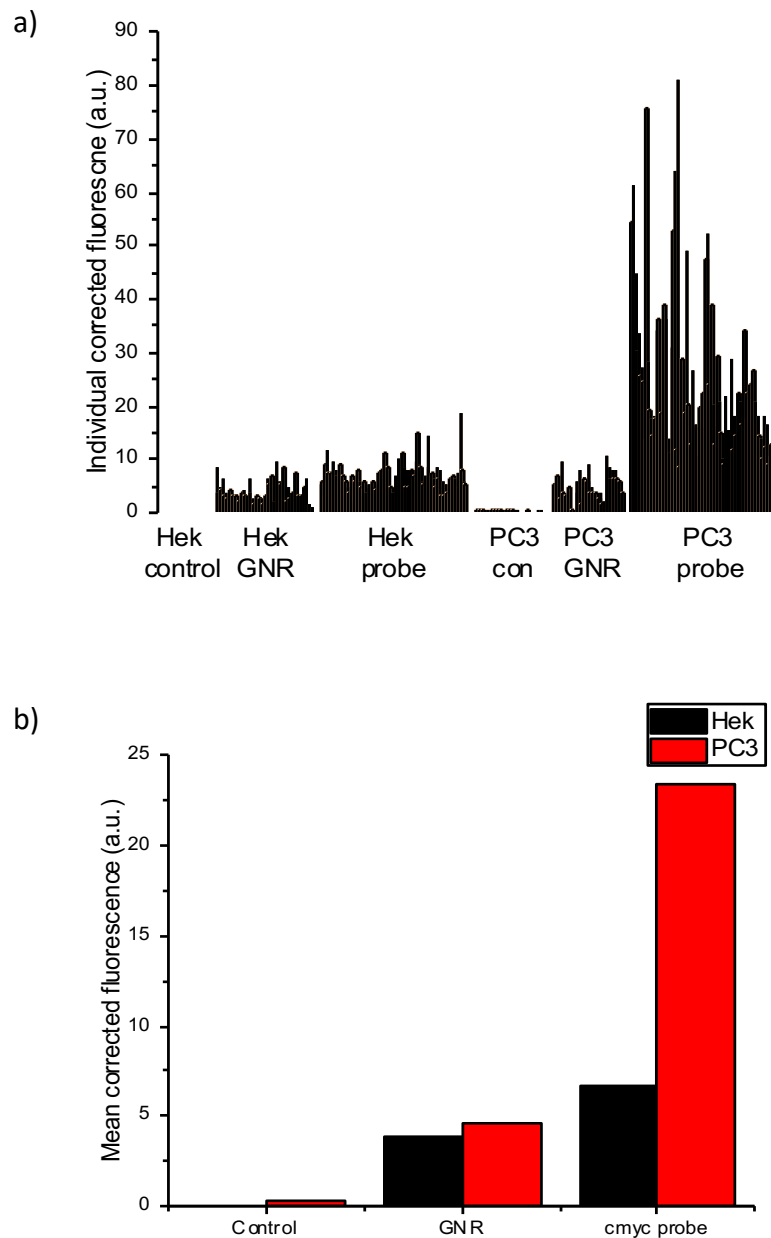


Figure 37. Fluorescence analysis of individual nanoprobes in Hek & PC3 cells.

a) Image j analysis was performed on the fluorescence intensity in individual spots in control, gold nanorod or nanoprobe treated Hek and PC3 cells. b) The average fluorescence intensity for control, gold nanorod or nanoprobe treated Hek and PC3 cells. Fluorescence imaging demonstrates that nanoprobe treated PC3 cells display over a 3 fold increase in fluorescence than nanoprobe treated Hek cells.

Individual spot analysis of nanoprobe within cells displayed a wide range of fluorescence intensities. This range of fluorescence could be due to the z positioning of nanoprobe within each cell. The confocal fluorescence images that were taken focus on one slice through the middle of each cell. However, nanoprobe can be uptaken in different position and can be found at different depths throughout the cell. Nanoprobe that are imaged out of focus will not display their full fluorescence intensity.

To investigate this, z-stack imaging was performed on PC3 cells after incubation with C-MYC nanoprobe. To perform this imaging, focusing was performed on the top and bottom of the cells of interest and z-stack slices were imaged at 1.5 μm increments (Figure 38). The emission of both DAPI (blue) and cy5 (red) were collected. Z-stack imaging shows that these PC3 cells have a high uptake of C-MYC nanoprobe and that nanoprobe are spread throughout the thickness of each cell. Some nanoprobe clusters can be seen around the inside of the cell membrane, as well as being around nuclear areas. White arrows indicate areas where the nanoprobe are intracellular. This further confirms that nanoprobe are capable of entering cells and can be localised to different areas within a cell. Furthermore, confocal imaging of individual z-planes in the centre of a cell results in the loss of data, as fluorescence cannot be collected from all nanoprobe.

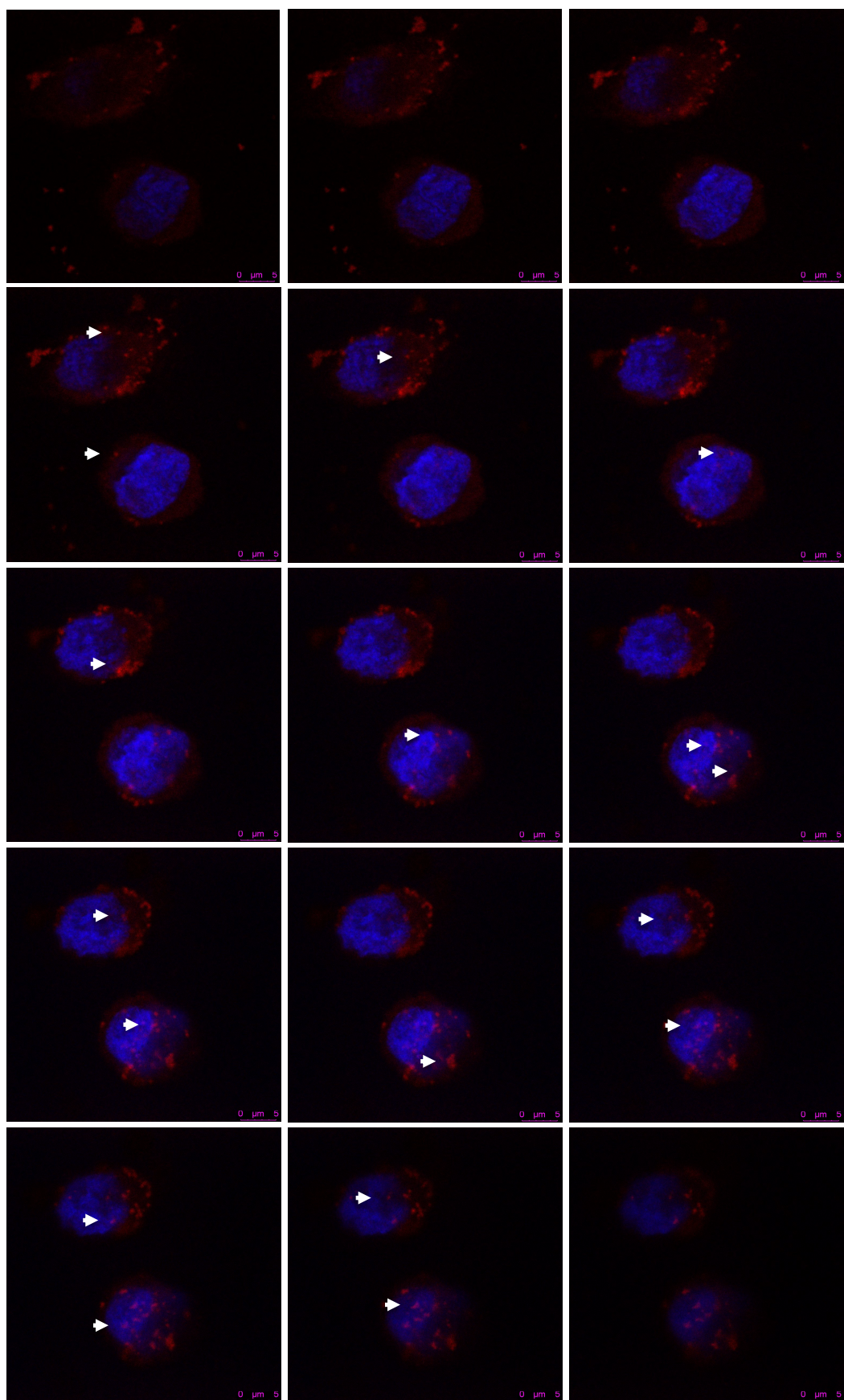


Figure 38. Z-stack of nanoprobes in PC3 cells.

Z-stack fluorescence imaging was performed on nanoprobe treated PC3 cells. Imaging of DAPI (blue) and cy5 from the nanoprobe (red) was performed at 1.5 μm increments. White arrows demonstrate areas where nanoprobes are intracellular.

6.3.2. Detection of biomarker mRNA in cells by fluorescence lifetime imaging microscopy

Two-photon luminance imaging was performed with excitation at 800 nm using a chameleon laser and emission was collected with a 685 nm short pass filter with an IR cut-off filter. Figure 39 displays the two-photon luminescence intensity image (left) and the corresponding fluorescence lifetime image (right) for gold nanorods alone (a), Hek control (b), Hek incubated with gold nanorods (c), Hek incubated with C-MYC nanoprobe (d) and PC3 control (e), PC3 incubated with gold nanorods (f) and PC3 incubated with C-MYC nanoprobe (g). Within each fluorescence lifetime image, the colour of each pixel depicts the different fluorescence lifetimes that are present, with the coloured scale bar depicting the automated lifetime range of each image in picoseconds.

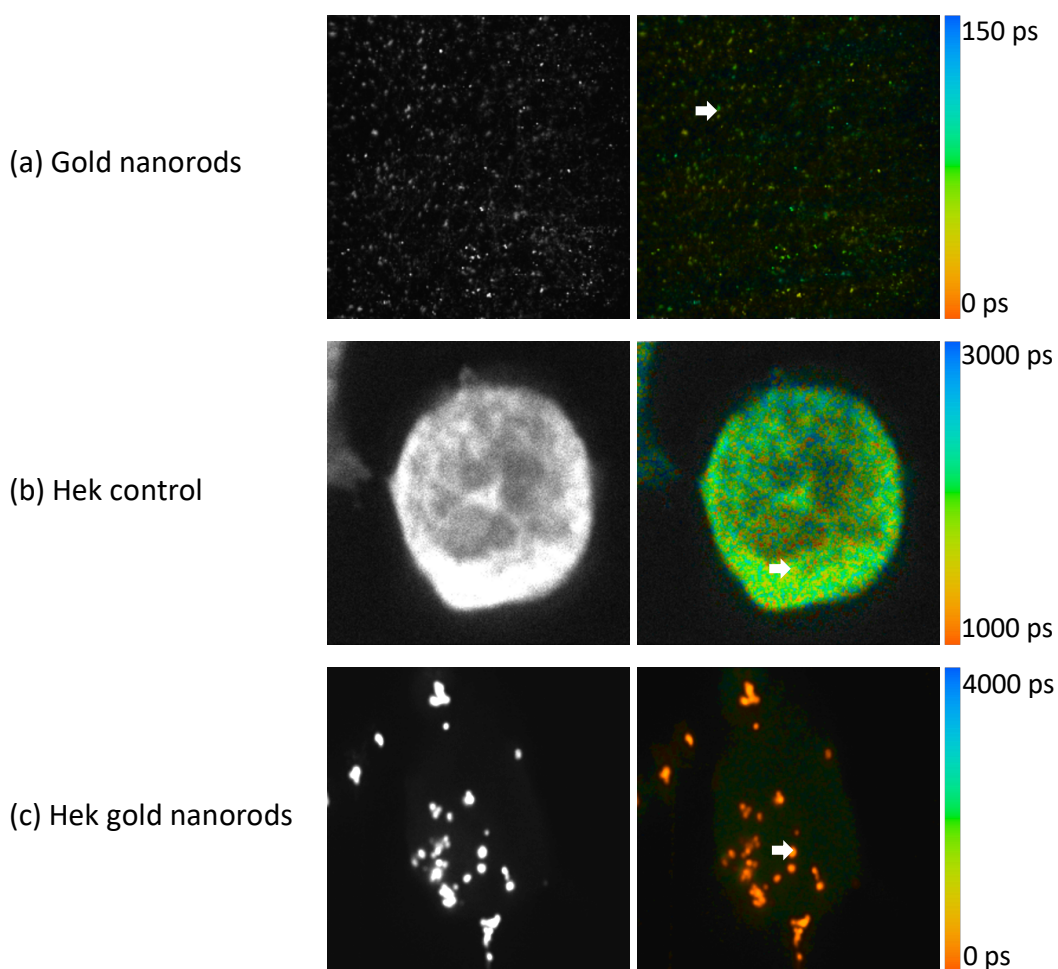


Figure 39 continues on the next page...

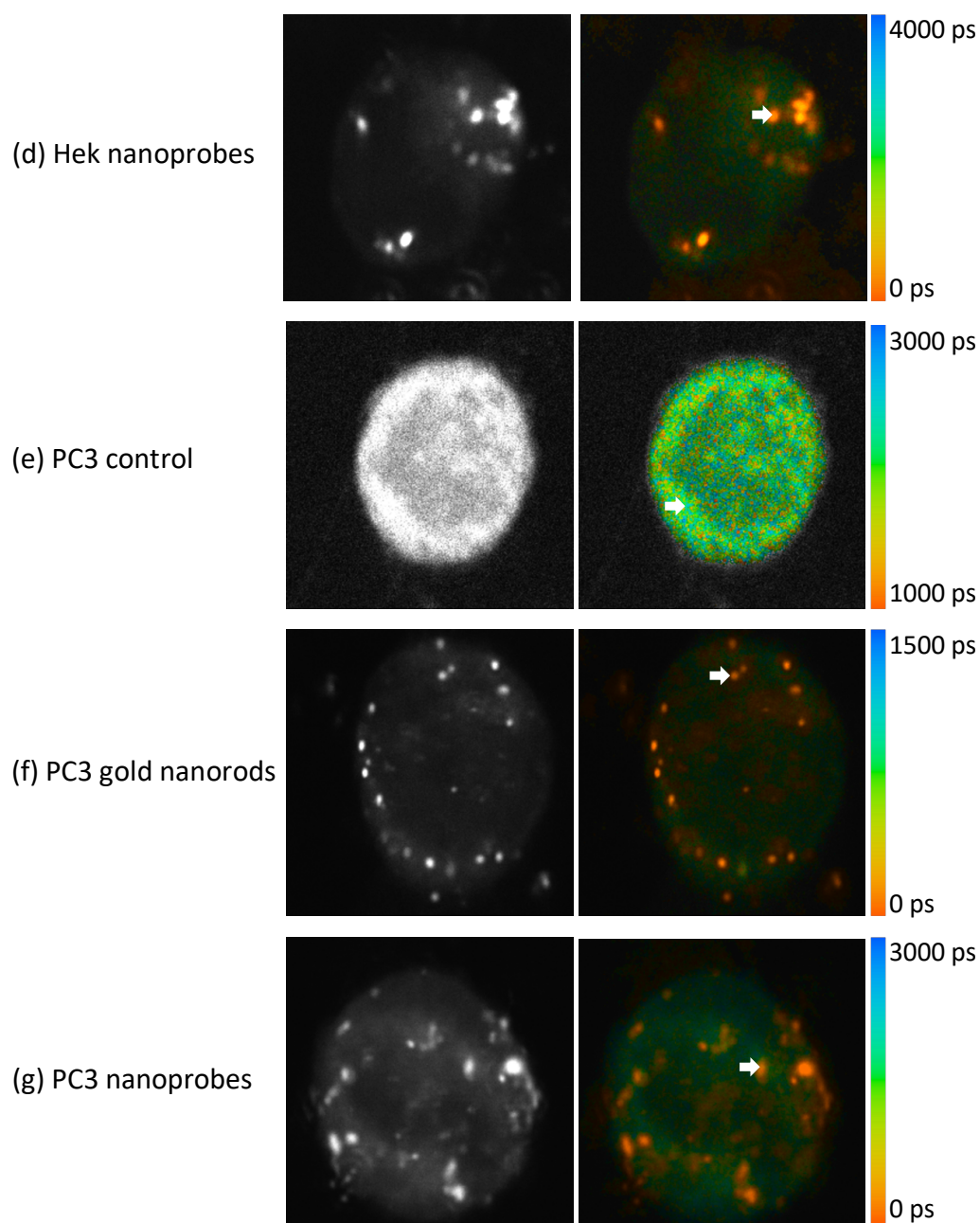


Figure 39. Fluorescence lifetime imaging of gold nanorods and nanoprobe in Hek & PC3 cells.

FLIM was performed on a slide of gold nanorods alone and on control, gold nanorod or nanoprobe treated Hek and PC3 cells. The intensity image (left) and lifetime image (right) are shown for each condition, with a fluorescence lifetime distribution colour scale bar. The white arrows represent positions for individual pixel lifetime analysis (Table 7).

Sample	τ_1	τ_2
GNR	67.5 ps	-
Hek control	1900.5 ns	-
Hek GNR	77.4 ps	-
Hek nanoprobe	58.1 ps	321.1 ps
PC3 control	2095.9 ps	-
PC3 GNR	71.7 ps	-
PC3 nanoprobe	79 ps	1065.5 ps

Table 7. Fluorescence lifetime values for individual pixel FLIM analysis.

Table 7 lists the lifetime decay values using a single or bi-exponential decay model for individual pixels within each image shown in Figure 39. A white arrow depicts the area of analysis for each image. FLIM of GNRs alone demonstrates a very short lifetime, which is a result of TPL of GNRs, and ranges between 50 and 100 ps with a single exponential decay (Figure 39a). The <100 ps recorded from GNRs is a result of the limit of the TCSPC system response time. The lifetime analysis of control cell images demonstrates that cellular auto-fluorescence has a fluorescence lifetime of around 2 ns with a single exponential decay (Figure 39b & e). In cells treated with GNRs, the orange pixels are considered to be areas that contain gold. Using a single exponential decay model on these pixels, a lifetime component is shown with a very short lifetime. This corresponds to the TPL of GNRs, with a lifetime of ~70 ps (Figure 39c & f).

Finally, Figure 39 d & g show Hek and PC3 cells that have been incubated with C-MYC nanoprobe, respectively. Pixels containing nanoprobe (orange dots) were fitted with a two-exponential decay model, so that the lifetime of both the GNRs and cy5 could be extracted. This analysis showed two lifetime components for each cell type. For individual pixel analysis, these lifetime values were 58 ps and 321 ps

for Hek, and 79 ps and 1065 ps for PC3. The first component in each cell line was the short lifetime component for the TPL of GNRs, which has previously been shown to be <100 ps. The second component, which represents cy5 within the nanoprobe, differed significantly between cell lines. In Hek cells, the fluorescence decay of cy5 was 321 ps, while in PC3 cells this was 1065 ps. The reduction in cy5 lifetime in Hek cells is due to fluorescence quenching of cy5 in the nanoprobe closed state, as Hek cells do not have a high expression of C-MYC. The fluorescence lifetime of cy5 was recovered in PC3 cells, which express high levels of C-MYC mRNA, due to the hybridization of nanoprobe to target mRNA. Nanoprobe binding to target mRNA results in hpDNA opening and the removal of fluorescence quenching.

This difference in the second lifetime components is observed following multiple FLIM image analysis. Figure 40 shows the lifetime values that were extracted from multiple single pixel analysis from FLIM images from 9 different Hek cells and 9 different PC3 cells. For each image, the first lifetime component is from GNRs and shows a very short lifetime of <100 ps. However, there was a significant difference in the second lifetime component, which represents cy5, in the Hek cells compared to PC3 cells. Hek cells are a non-cancer cell line that have low expression of the nanoprobe target C-MYC. Therefore, when C-MYC nanoprobe are incubated in Hek cells the hpDNA will have a low concentration of target mRNA to bind to, therefore the majority of nanoprobe will remain in the hpDNA conformation and the majority of cy5 fluorophores will be quenched. This is represented by a second fluorescence lifetime components of 200-600 ps in Hek cells.

Conversely, PC3 is a cancer cell line that over-expresses C-MYC. Therefore, when the nanoprobe is incubated in these cells, there will be a high expression of the mRNA target which the nanoprobe can bind to, resulting in the dissociation of the hpDNA structure, removal of fluorescence quenching and restoration of the fluorescence of cy5. This is represented by an increase in the lifetime of the second component in PC3 cells, which ranges from 600- 1200 ps. The average lifetime of free cy5 is ~1 ns, however the fluorescence lifetime of a fluorophore can vary

greatly depending on the local environment. Therefore, the second lifetime component in PC3 cells represents the lifetime of un-quenched cy5.

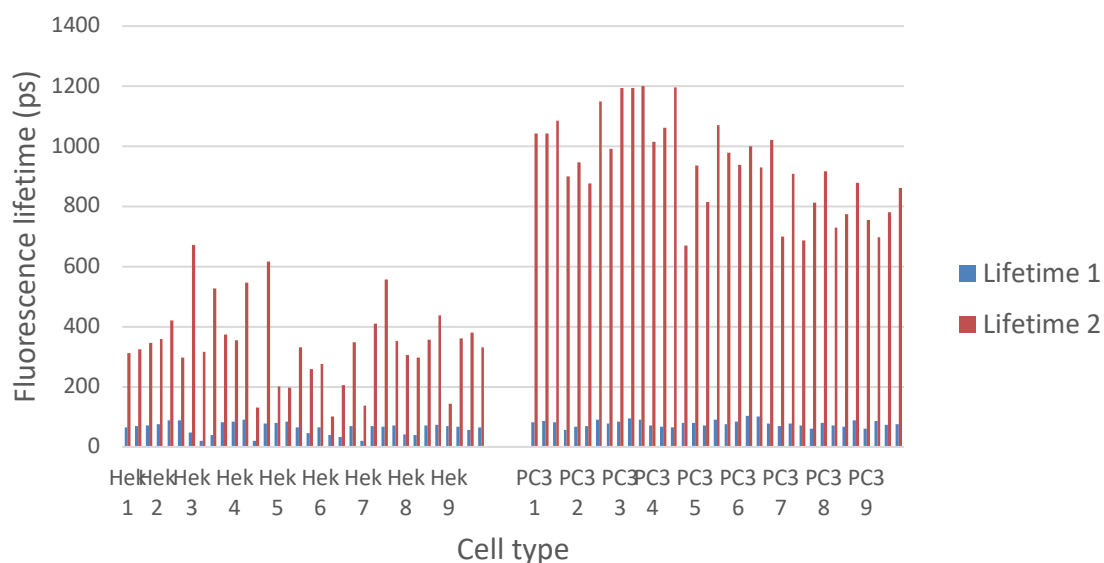


Figure 40. Lifetime values of c-myc nanoprobe in Hek & PC3 cells.

Multiple single pixels were analysed by a two-exponential decay model in Hek and PC3 cells. This is represented by two lifetime components; the first lifetime represents gold nanorods which display a short lifetime of <100 ps. The second lifetime component represents cy5. This ranges from 200- 600 ps in Hek cells, due to fluorescence quenching in the closed hpDNA design. In PC3 cells, which express high levels of c-myc, the second lifetime component increases and ranges between 600 – 1200 ps. This is due to binding to target mRNA, which opens the hpDNA and removes fluorescence quenching.

6.3.3. FLIM image analysis of nanoprobe in cells

FLIM analysis was performed on the same images as shown in Figure 39 with a different method. A MATLAB code was developed in our lab that calculates the fluorescence lifetime of pixels and generates a lifetime map and a phasor plot analysis. The fluorescence lifetime for each pixel is calculated using a centre-of-mass algorithm, described in Equation 9.

$$\cong \tau_{MMM} = \left(\frac{\sum_{j=0}^{M-1} jN_j}{N_c} + \frac{1}{2} \right) h,$$

Equation 9.

During the FLIM measurement, the time to digital converter records lifetimes into M time bins, with the bin width of h . N_j is the number of recorded events in the j^{th} time bin, while N_c is the total signal count within the measurement window (203). To calculate the average lifetime of each FLIM image or area of interest, the decay histograms for each individual pixel are merged and the average lifetime is calculated using the centre-of-mass method.

The phasor plot is constructed using two phasor vectors, G and S , where each vector is calculated as shown in Equation 10.

$$g_{x,y}(\omega) = m_{x,y} \cos(\varphi_{x,y})$$

$$s_{x,y}(\omega) = m_{x,y} \sin(\varphi_{x,y})$$

Equation 10.

In this calculation, $m_{x,y}$ and $\varphi_{x,y}$ are the modulation ratio and the phase delay measured given a particular modulation frequency (ω) at a specific pixel location (x,y) (204). Using these vectors, the lifetime of any single exponential decay is displayed within a semicircle curve, that is known as the universal circle, and is centred at $g = 0.5$, $s = 0$. Phasor plots transform the histogram of lifetime decays

into a vector, where each pixel in the image provides a point in the phasor plot. Longer lifetimes are depicted on the top left of the phasor plot, while shorter lifetimes are depicted to the bottom right of the phasor plot (Figure 41).

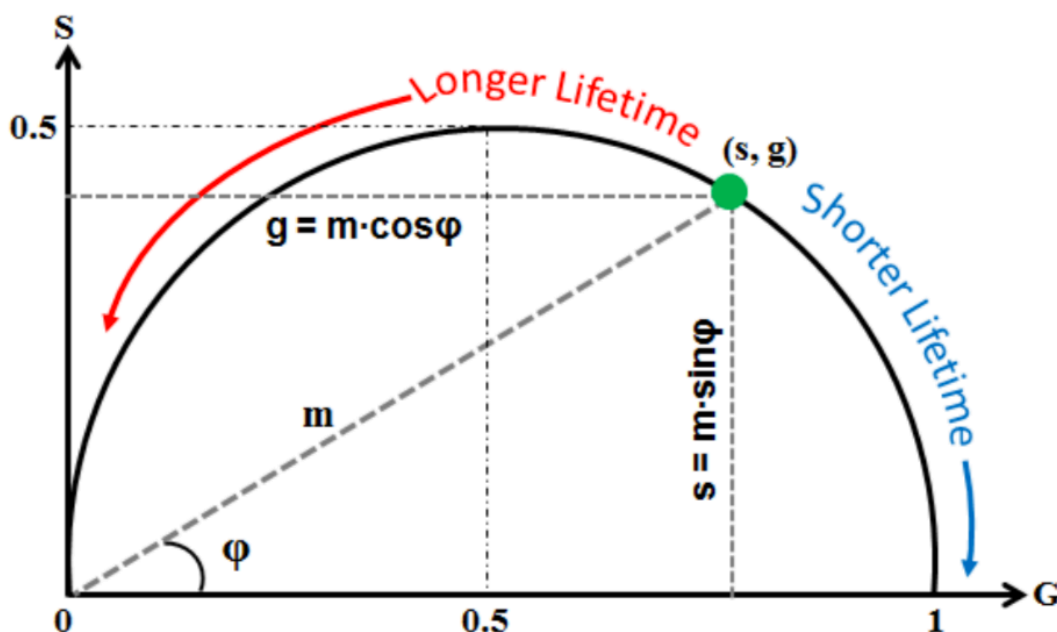


Figure 41. The phasor plot vector space.

Within a phasor plot, a lifetime decay component is represented by a point within the universal circle. Figure was used with permissions from ISS © Copyright 2014.

Analysing FLIM by phasor plots provides a global view of the fluorescence decay across a whole image or a region of interest. Furthermore, the presence of different molecular species can be visually recognised by their difference in lifetime decay values (205). Another advantage of this analysis is the ability to select the lifetime and intensity values to plot in the analysis and individual areas within the FLIM image can be analysed in detail.

Figure 42 depicts FLIM images and phasor plots of a) GNRs, b) Hek control, c) Hek incubated with GNRs, d) Hek incubated with C-MYC nanoprobe, e) PC3 control, f)

PC3 incubated with GNRs and g) PC3 incubated with C-MYC nanoprobe. Fluorescence lifetime image maps were created across cells with 256 x 256 pixels. The lifetime analysis was performed on the GNR sample between 0 ns and 0.2 ns. This shows that the GNR dots on the image exhibit very short lifetimes and the phasor plot shows a population at the bottom right of the curve, which further confirms the short lifetime of this sample. The lifetime analysis on all cell samples was performed between 0 ns to 3 ns. The lifetime colour scale is located on the right of each FLIM image, with black pixels depicting the background where no lifetimes were calculated.

The control Hek and PC3 images both exhibit long lifetimes with an average lifetime of 2.48 ns and 2.9 ns, respectively. This long fluorescence lifetime represents cellular autofluorescence and the phasor plots depict a population at the top left of the curve. In samples incubated with GNRs, red dots can be visualised within the cell, which depicts areas of GNRs with very short lifetimes. In the phasor plot analysis, a tail can be visualised from the cell autofluorescence signal, to the GNR signal. The average fluorescence lifetime of the Hek and PC3 cell image with GNRs was 0.54 ns and 1.59 ns, respectively. In cells incubated with nanoprobes, red dots can again be visualised that represent areas that contain GNRs. Compared to GNR incubation alone, it appears that areas that contain nanoprobe are associated with a higher proportion of longer lifetime components of yellow and green. The average lifetime for the image of the Hek and PC3 cell incubated with nanoprobes was 1.08 ns and 1.8 ns, respectively. However, in both the GNR and nanoprobe treated cells, pixels corresponding to the cell autofluorescence are contributing to this signal. In the phasor plot analysis of nanoprobe treated cells, the cell autofluorescence population is visualised as well as a tail leading to the GNR signal. However, as compared to the phasor plot of cells incubated with GNRs, the phasor plot of cells incubated with nanoprobes display a wider tail, depicting the presence of a longer lifetime that is between the lifetime decay of GNRs and cell autofluorescence.

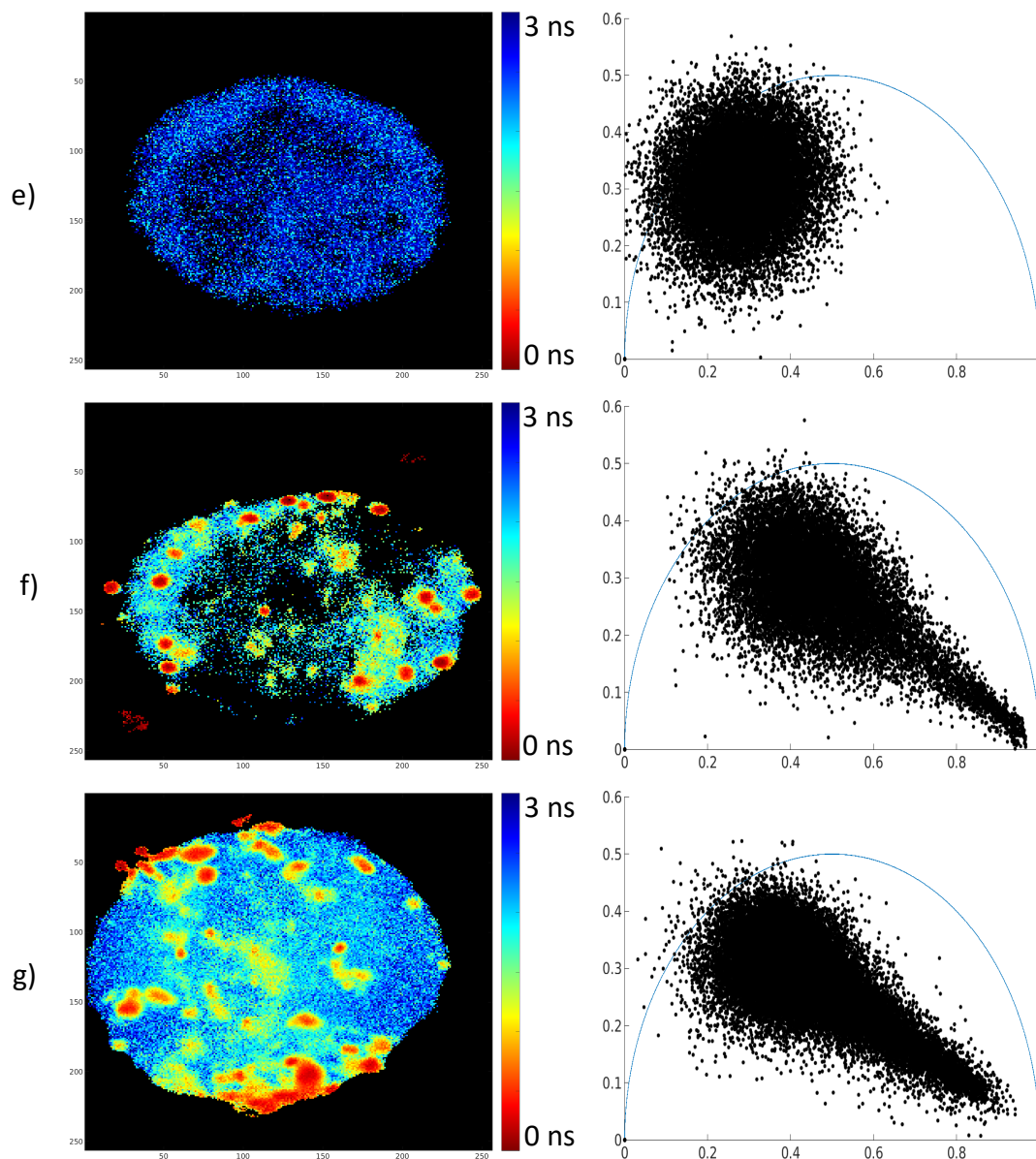


Figure 42. Fluorescence lifetime analysis of GNRs and nanoprobe in cells.

The fluorescence lifetime of a) GNRs alone, b) Hek cell control, c) Hek cell incubated with GNRs d) Hek cell incubated with C-MYC nanoprobe, e) PC3 cell control, f) PC3 cell incubated with GNRs and g) PC3 cell incubated with C-MYC nanoprobe. Fluorescence lifetimes were analysed between 0-2.5 ns by FLIM image map (left) and by phasor plot (right). This analysis shows GNRs alone display a population with short lifetimes in the phasor plot while control cells display a population with a long fluorescence lifetime of cell autofluorescence. Cells incubated with GNRs display a range of lifetimes, from the GNR population to the cell autofluorescence population. Cells incubated with nanoprobe appear to have an extra lifetime component in the middle of the phasor plot, which demonstrates the fluorescence lifetime of cy5.

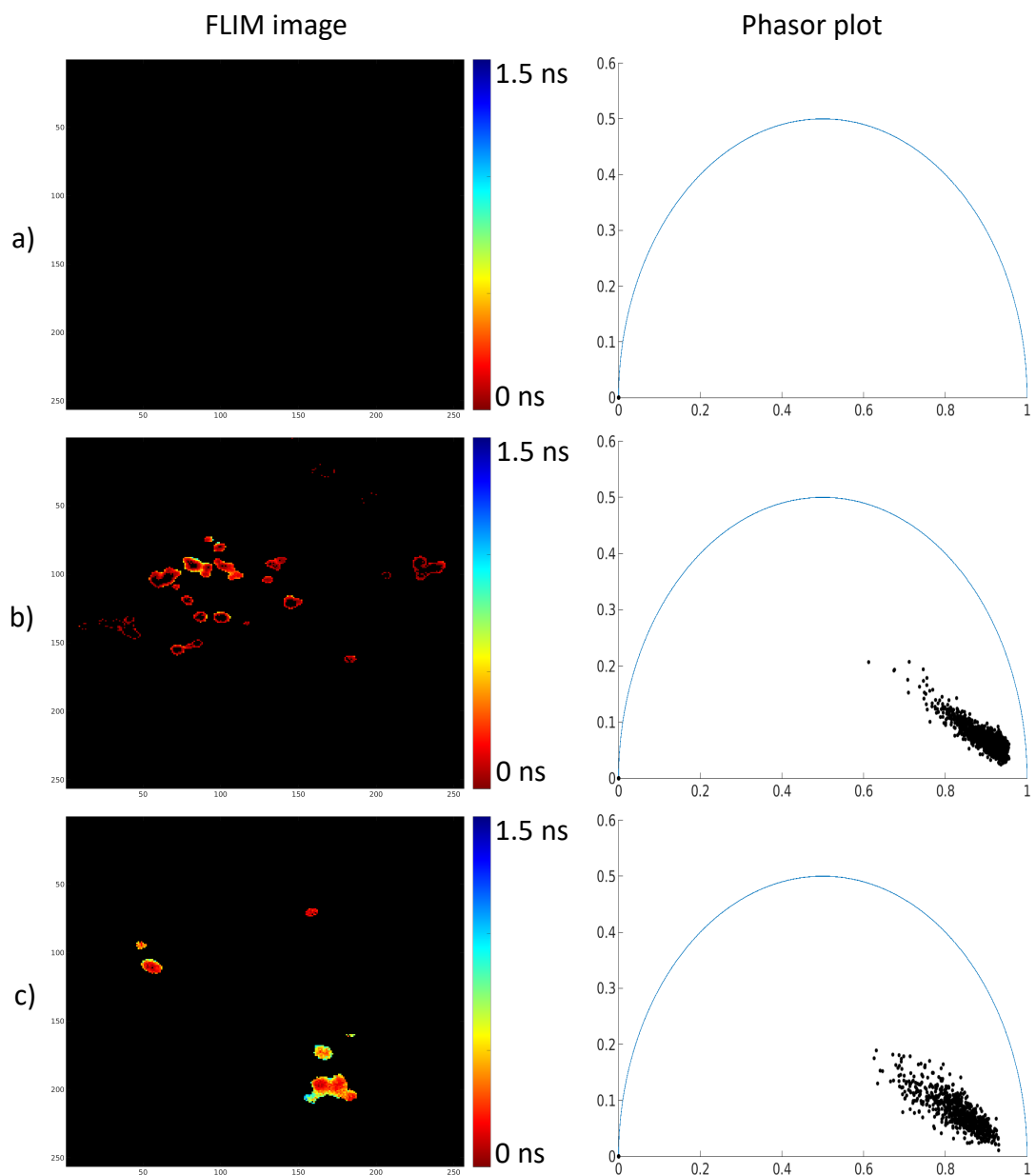
In order to perform a more detailed analysis on the lifetime contribution from GNRs and nanoprobes, the same cell images were analysed with a fluorescence lifetime range of 0-1.5 ns, as the lifetime of cy5 in the quenched and unquenched state previously measured varied from 0.3- 1.2 ns and to remove the long lifetime contribution from cell autofluorescence, which ranges from 1.7 – 3.5 ns in the control cell analysis. Figure 43 depicts the analysis of Hek control, GNR and nanoprobe incubation and PC3 control, GNR and nanoprobe incubated cells.

In the control Hek and PC3 cells (Figure 43 a & d), when cell autofluorescence is removed, there are no pixels detected with a lifetime value of between 0-1.5 ns and the phasor plot is empty. In Hek cells incubated with GNRs (Figure 43 b), the FLIM image shows red/yellow pixels with the image average lifetime of 0.27 ns, depicting the short lifetime contribution from GNRs. In Hek cells incubated with nanoprobes (Figure 43 c), the FLIM image shows red, yellow and green pixels, with the average lifetime of this image of 0.58 ns. This shows that the lifetime of the nanoprobe in Hek cells is longer than the lifetime of GNRs alone in Hek cells. In both GNR and nanoprobe incubated Hek cells, the phasor plot shows a population at the bottom right of the graph, demonstrating the main lifetime contribution from the images is from GNRs.

In PC3 cells incubated with GNRs (Figure 43 e), the FLIM image depicts red/yellow pixels where GNRs are located and the average lifetime of this image is 0.49 ns. The phasor plot depicts a population at the bottom right of the plot, which is very similar to the phasor plot of Hek cells incubated with GNRs and depicts the short lifetime contribution from GNRs. In PC3 cells incubated with nanoprobes (Figure 43 f), the FLIM image demonstrates pixels ranging from red-blue, with the image showing an average lifetime of 1.11 ns. Interestingly, the phasor plot of PC3 nanoprobes is very different from GNR incubated Hek and PC3 cells and nanoprobe incubated Hek cells, with a lifetime population ranging from the bottom left to the top right of the phasor plot. This demonstrates the presence of a longer lifetime

contribution, and as the cell autofluorescence has been removed, this contribution is likely to be from unquenched cy5 within the nanoprobe.

The combined analysis of FLIM images with phasor plot lifetime distribution has shown that in Hek cells incubated with C-MYC nanoprobe, which have low expression of the mRNA target C-MYC, the average lifetime increased as compared to GNR incubation, but the population was still primarily short lifetimes, as cy5 was quenched. In PC3 cells, which overexpress the mRNA target C-MYC, the average lifetime of the nanoprobe greatly increased, and the phasor plot revealed the presence of a long lifetime components, which demonstrates the nanoprobe binding to target mRNA and the removal of fluorescence quenching of cy5.



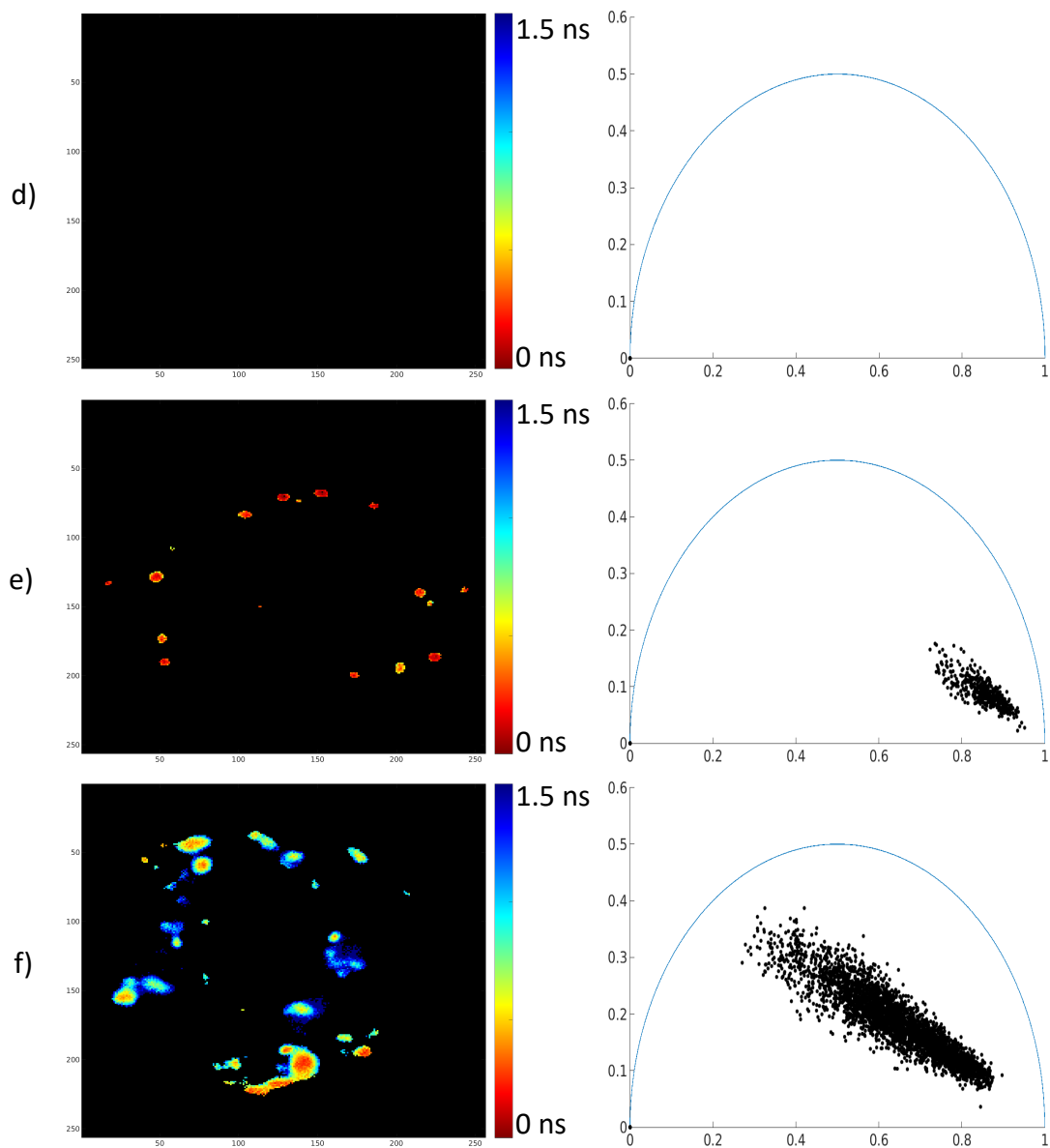


Figure 43. Fluorescence lifetime analysis of only the GNR and nanoprobe contribution in cells.

The fluorescence lifetimes of of a) Hek cell control, b) Hek cell incubated with GNRs c) Hek cell incubated with C-MYC nanoprobe, d) PC3 cell control, e) PC3 cell incubated with GNRs and f) PC3 cell incubated with C-MYC nanoprobe were analysed between 0-1.5 ns by FLIM image map (left) and by phasor plot (right). This analysis shows that control cells analysed in this lifetime range do not display any fluorescence lifetimes. Cells incubated with GNRs display a population with short lifetimes in the phasor plot and both Hek and PC3 cells show a similar population. The Hek cell incubated with nanoprobes displayed a similar phasor plot to GNR treated cells, with a short lifetime population. The PC3 cell incubated with nanoprobes display a population with longer lifetime components in the phasor plot, which demonstrates the fluorescence lifetime of cy5.

Finally, fluorescence lifetime analysis and phasor plot analysis were performed on individual intracellular areas that contained nanoprobe with a lifetime range of 0.3-1.5 ns to remove the contribution from both GNRs and cell autofluorescence and to only capture the signal contribution from cy5. Three areas were analysed in Hek cells and three areas in PC3 cells. For each selected nanoprobe area analysis, a pixel in the middle of an area containing nanoprobe was highlighted using the pixel selector function and an area of 10 x 10 pixels was selected from the mid-point. In each phasor plot analysis, the black spots represent the phasor plots from the pixels from the whole image, while the green spots represent the pixels in the highlighted image area.

In the nanoprobe area analysis in Hek cells (Figure 44), each FLIM map depicts an area made up of red to green pixels, with a lifetime range of 0.3 ns to around 0.9 ns. The average lifetime was calculated for each nanoprobe image and these were 0.47 ns for image 1, 0.75 ns for image 2 and 0.59 ns for image 3. In the phasor plot analysis for each individual nanoprobe the green dots demonstrate a population at the bottom right area of the curve, demonstrating the contribution from cy5 within the nanoprobe has a short lifetime in Hek cells.

In the nanoprobe area analysis in PC3 cells (Figure 45), each FLIM map depicts a red/yellow core, which depicts a lifetime component of around 0.5 ns, and a ring of blue pixels which represents lifetimes of between 1-1.5 ns. The average lifetime was calculated for each nanoprobe image and these were 1.06 ns for image 1, 1.34 ns for image 2 and 1.36 ns for image 3. Therefore, the average lifetime of nanoprobe in PC3 cells were much longer than the average lifetimes of nanoprobe in Hek cells, representing the removal of fluorescence quenching in C-MYC expressing cells. Furthermore, in the phasor plot analysis for each individual nanoprobe the green dots demonstrate a population in the middle area of the curve, demonstrating these nanoprobe exhibit a longer lifetime component, as compared to nanoprobe in Hek cells.

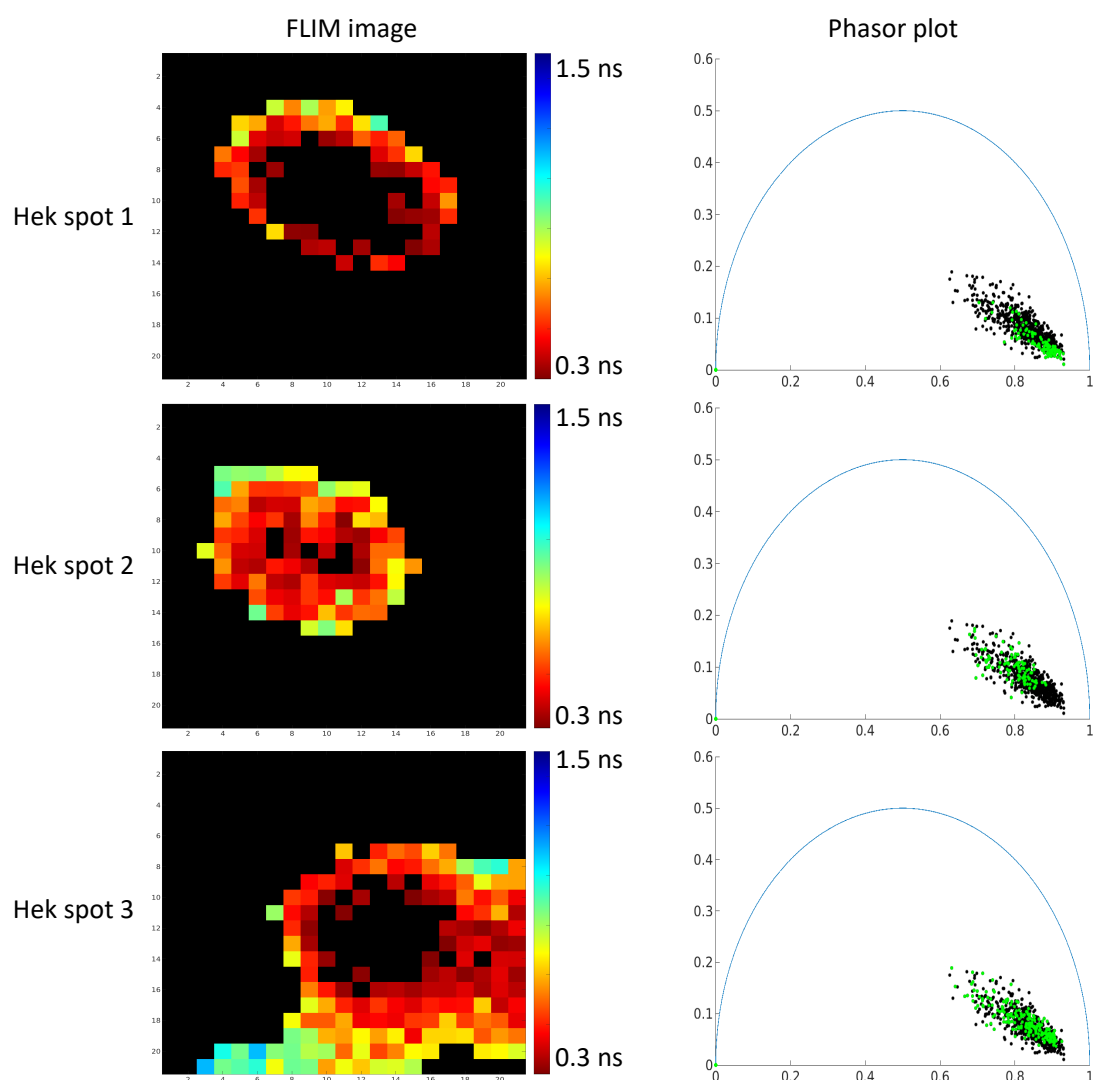


Figure 44. Fluorescence lifetime analysis of C-MYC nanoprobe areas in in Hek cells.

Three areas containing nanoprobe were analysed by fluorescence lifetime imaging map (left) which depicts the lifetime values of each pixel and phasor plot analysis (right). In each phasor plot analysis, the black dots represent the lifetime value of each pixel within the whole FLIM image, while green dots represent the lifetime value of each pixel within the highlighted nanoprobe area. All nanoprobe analysed in Hek cells displayed a population with low fluorescence lifetimes.

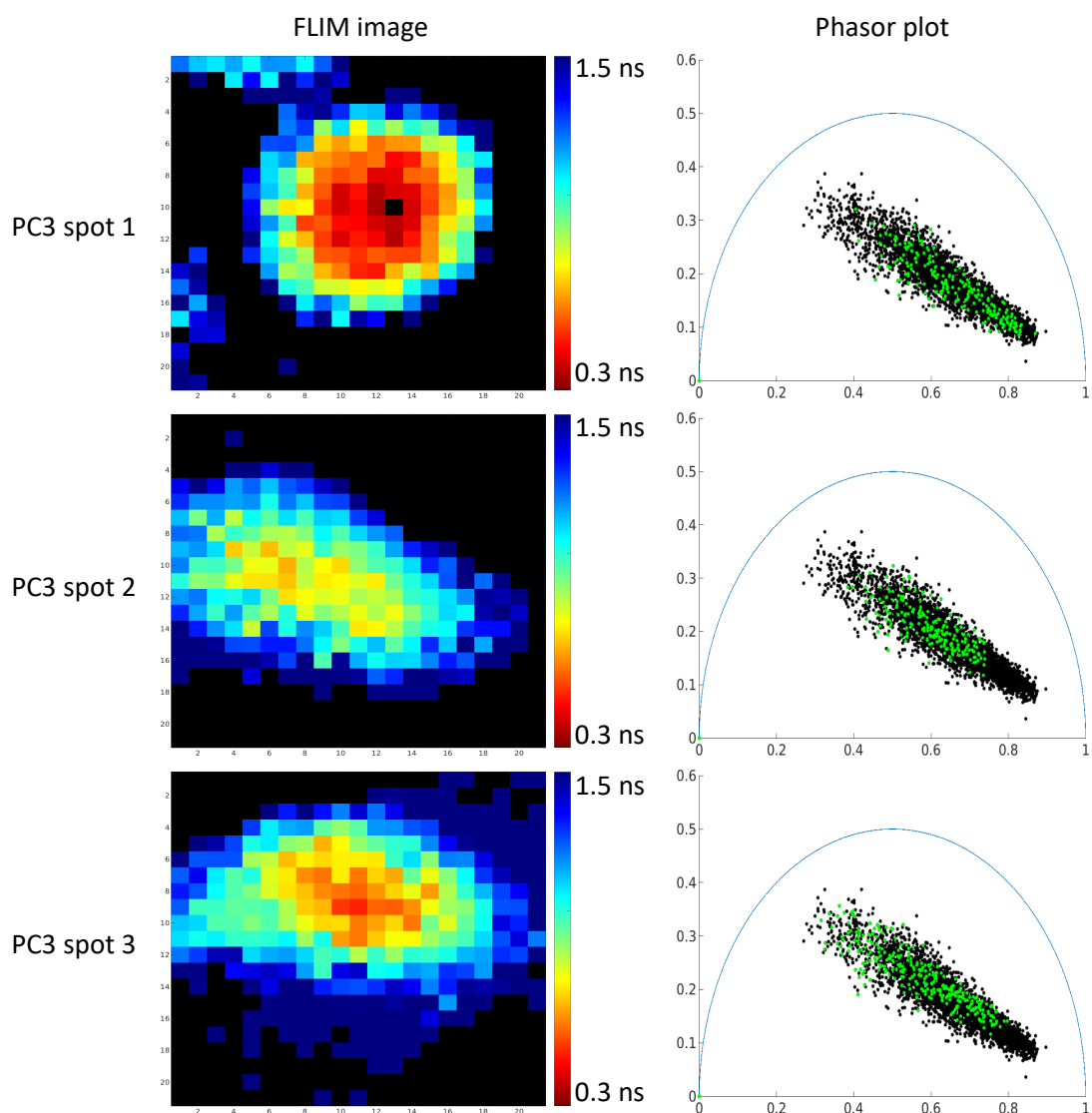


Figure 45. Fluorescence lifetime analysis of C-MYC nanoprobe areas in PC3 cells.

Three areas containing nanoprobe were analysed by fluorescence lifetime imaging map (left) which depicts the lifetime values of each pixel and phasor plot analysis (right). In each phasor plot analysis, the black dots represent the lifetime value of each pixel within the whole FLIM image, while green dots represent the lifetime value of each pixel within the highlighted nanoprobe area. All nanoprobe analysed in PC3 cells displayed a population with a range of fluorescence lifetimes and displays a population that has higher lifetimes than the nanoprobe in Hek cells.

This analysis provides a complimentary method of extracting fluorescence lifetime values from FLIM images. TSCPC individual pixel lifetime analysis and phasor plot lifetime analysis have both demonstrated that C-MYC nanoprobe specifically bind to target mRNA and produce a cy5 fluorescence signals in C-MYC overexpressing cancer cells.

6.3.4. Ability to differentiate quenched and unquenched cy5 within the nanoprobe by FLIM

FLIM has demonstrated the ability to detect target mRNA in single cells based on the fluorescence lifetime values of the nanoprobe. To ensure the differences in fluorescence lifetime of the nanoprobe are not due to imaging artifacts or selection of pixels, both Hek and PC3 cells were incubated with either nanoprobe or nanoprobe pretreated with cDNA (nanoprobe-cDNA). This was to investigate whether the fluorescence lifetime of cy5 could be discriminated in Hek cells incubated with either nanoprobe or nanoprobe-cDNA.

The fluorescence emission of the C-MYC nanoprobe alone (black) and following incubation with cDNA (red), is shown in Figure 46. This shows that in solution phase, nanoprobe display a low background fluorescence when the hpDNA is in the closed formation, as cy5 is in close proximity to the GNR surface and is in a quenched state. Following a 2-hour incubation with cDNA, the fluorescence emission showed a 4-fold increase. This demonstrates that after cDNA incubation and target binding, the nanoprobe is in the open state, where hpDNA is opened and cy5 is no longer quenched by the gold.

Hek and PC3 cells were incubated with 0.1 nM of nanoprobe or nanoprobe-cDNA for 6 hours before slides were prepared. FLIM was then performed on nanoprobe treated and nanoprobe-cDNA treated individual Hek and PC3 cells. Three FLIM images were taken for each condition and pixels containing nanoprobe were analysed with a two-exponential decay model. Figure 47 shows the intensity and lifetime images for one image for each sample. Single pixel analysis was performed

with a two-exponential decay fit on nanoprobe areas for each image, with the white arrow depicting the position of the analysis.

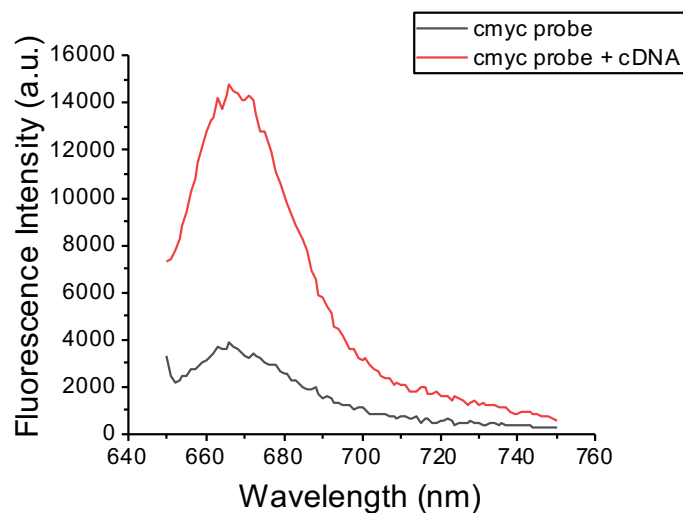


Figure 46. Fluorescence emission of the C-MYC nanoprobe with nanoprobe alone and nanoprobe treated with cDNA.

Hek and PC3 cells were incubated with nanoprobe with closed hpDNA (black line) or nanoprobe with open hpDNA after cDNA incubation (red line).

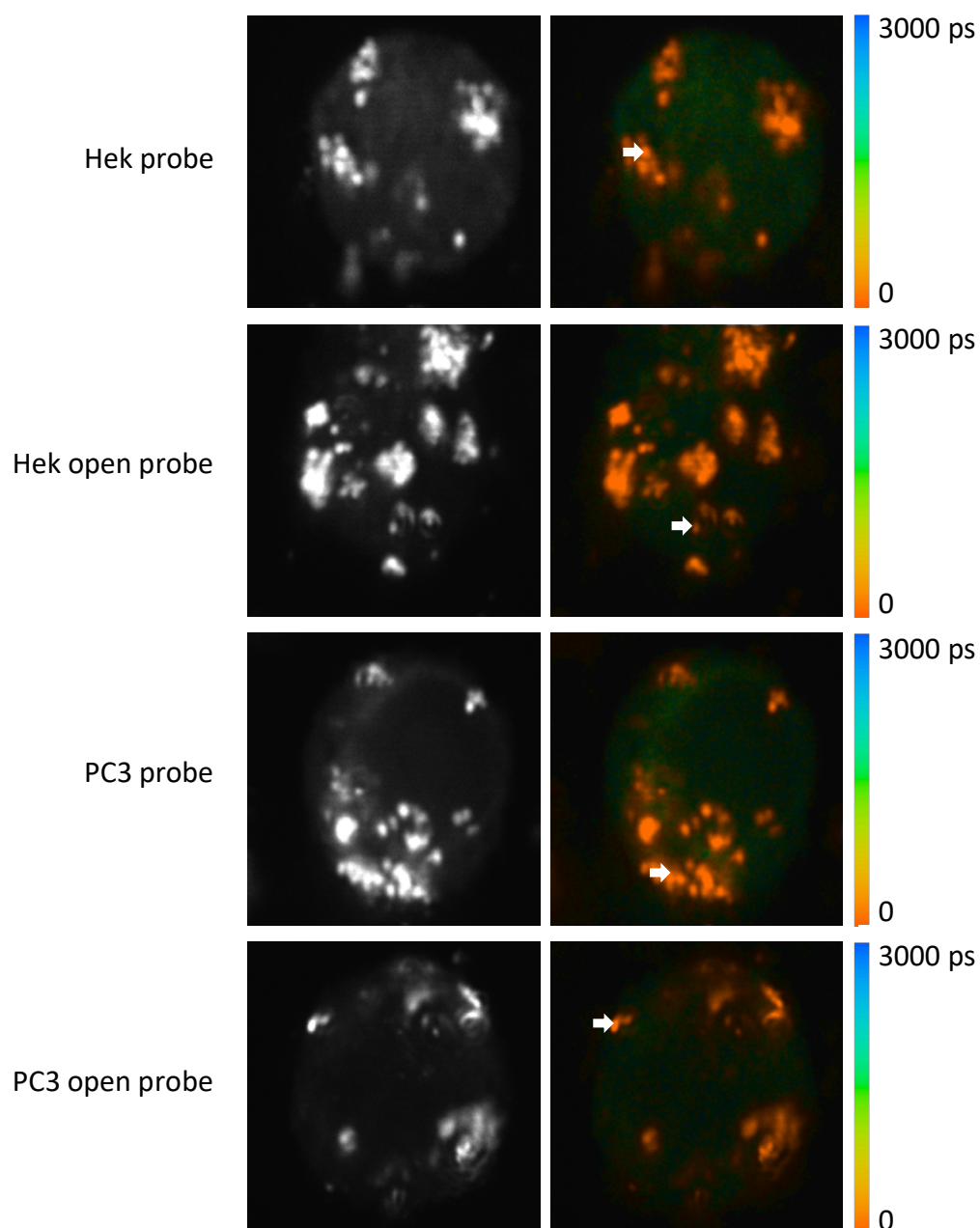


Figure 47. Fluorescence lifetime imaging of nanoprobe and nanoprobe-cDNA in Hek and PC3 cells.

FLIM of Hek and PC3 cells incubated with nanoprobe alone or nanoprobe-cDNA. Images on the left are intensity and images on the right are fluorescence lifetime distribution images. White arrows indicate the location for individual pixels analysis for two-exponential lifetime decay fittings.

Sample	τ_1	τ_2
Hek probe	65.1 ps	297 ps
Hek open probe	99 ps	745.4 ps
PC3 probe	72.1 ps	755.7 ps
PC3 open probe	76.9 ps	744.5 ps

Table 8. Fluorescence lifetime values from individual pixels from FLIM of open and closed hpDNA in Hek and PC3 cells.

Table 8 displays the lifetime values for single pixel analysis in each FLIM image, with the position of pixel analysis depicted by white arrows in Figure 47. This analysis shows that in Hek cells treated with nanoprobe the fluorescence lifetime is the same as previously shown, with the first lifetime component being very short of <100 ps, demonstrating the TPL of GNRs, and a second lifetime exists at around 0.3 ns, which represents the fluorescence lifetime of quenched cy5. When Hek cells are incubated with a nanoprobe-cDNA, the second lifetime component has a much longer lifetime of around 0.75 ns. This shows that the fluorescence lifetime of cy5 in the open hpDNA design has increased, as the fluorescence quenching has been removed due to the opening of the hpDNA. PC3 cells with nanoprobe and nanoprobe-cDNA display very similar fluorescence lifetime components, with the first lifetime component being the scattering of GNRs at <100 ps and the second lifetime component exhibiting a decay of around 0.75 ns, demonstrating the reduction of fluorescence quenching of cy5 as the nanoprobe hpDNA conformation is opened in both samples.

As well as the single pixel analysis shown in Table 8, fluorescence lifetime analyses for Hek and PC3 cells incubated with nanoprobe and nanoprobe-cDNA was performed on multiple pixels across three cell images taken for each condition. The lifetime distribution values for nanoprobe in Hek and PC3 cells are shown in Figure 48 a&b, respectively.

This analysis was performed across three different cells and in different pixels that contained the nanoprobe. For each pixel, a two-exponential decay fit was performed. The first lifetime component was similar across both cell types and both open and closed hpDNA. This was a lifetime component of <100 ps, which represents the TPL of GNRs. In Hek cells incubated with nanoprobe alone, the second lifetime component across 3 cells was between 150 – 450 ps, which represent quenched cy5 in the nanoprobe. In Hek cells incubated with nanoprobe-cDNA, the second lifetime component was found to be between 650 – 900 ps. This increase in fluorescence lifetime represents the removal of the quenching of cy5 from the GNRs, as the hpDNA has been opened before being incubated with the cells. In PC3 cells incubated with nanoprobe, the second fluorescence lifetime component ranges from 550 – 900 ps and there was not a clear difference in the second lifetime component in PC3 cells incubated with nanoprobe-cDNA, which ranged from 520 – 970 ps. This demonstrates that the nanoprobe does not open randomly in cells that don't express the target and that the fluorescence lifetime of the fluorophore in the nanoprobe is a good indication whether it is bound to a target. This also confirms that fluorescence lifetime is a robust technique to determine the hybridisation status of the nanoprobe, which represents the expression of biomarker mRNA.

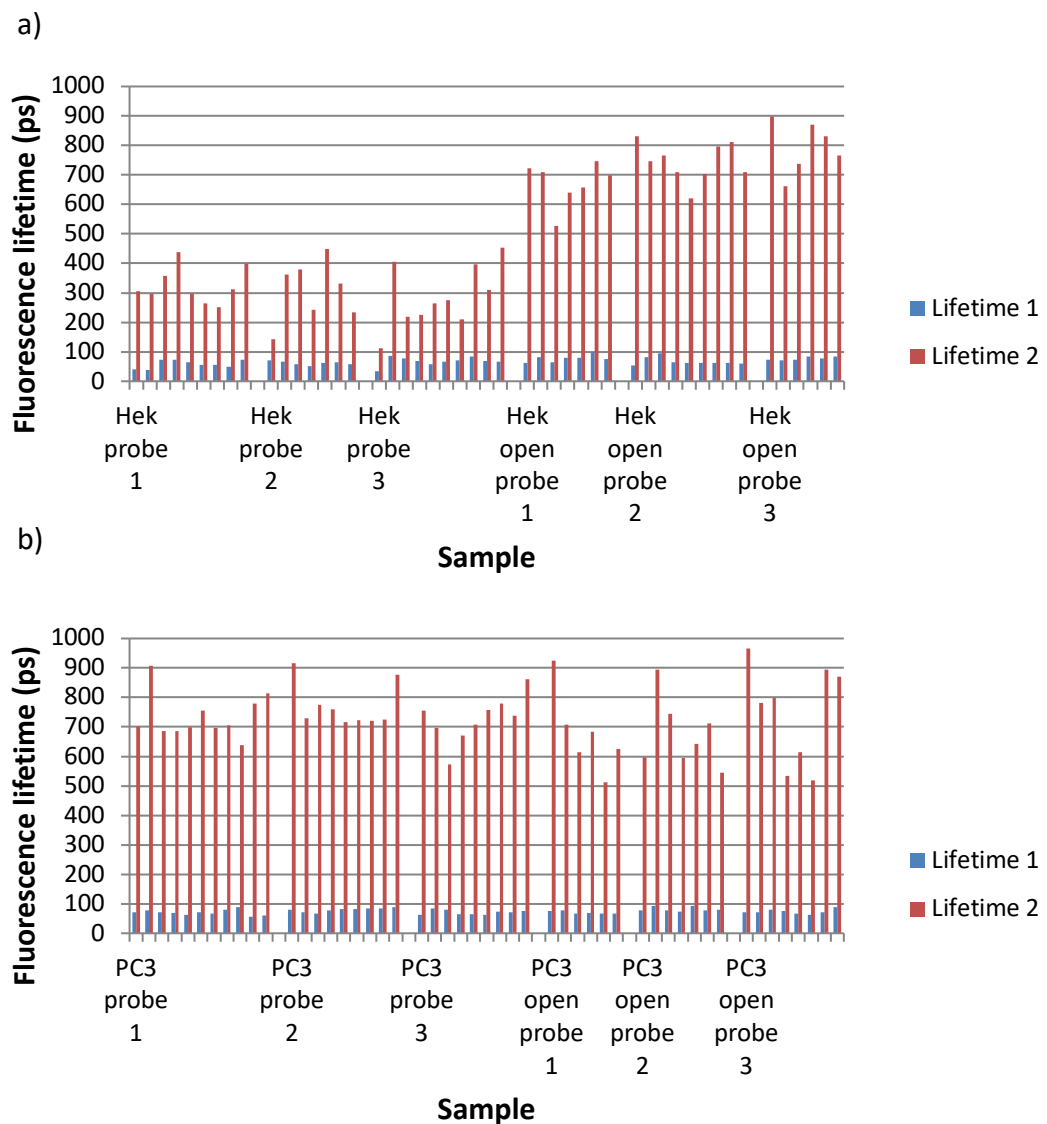


Figure 48. FLIM analysis of open and closed nanoprobe in Hek and PC3 cells.

Lifetime analysis of individual pixels in a) Hek cells and b) PC3 cells that have been incubated with nanoprobe or nanoprobe incubated with cDNA. This demonstrates that the nanoprobe fluorescence is a result of binding to target mRNA in target expressing cancer cells.

The range of fluorescence lifetimes of cy5 detected in the open hpDNA samples are slightly lower than the range of cy5 fluorescence lifetimes detected in the first set of FLIM images. This could be due to the 3D positioning of the nanoprobe within the cell and the extent of focusing on individual nanoprobe clusters, as clusters in sharp focus would give a more reliable lifetime analysis than pixels out of focus. This could also be due to the extent of DNA opening and the packing density of DNA on the GNR surface. Previous studies have shown that not all hpDNAs can open in response to mRNA target, as opened hpDNAs can block more target from binding to neighbouring hpDNAs (206). Therefore, if the packing density of hpDNA on the GNR surface is very high, then a smaller proportion of hpDNA molecules are free to open upon target binding. This results in a proportion of cy5 within the nanoprobe to be quenched; therefore, the average fluorescence lifetime of each nanoprobe would be reduced.

The combination of single pixel lifetime analysis and lifetime mapping analysis of FLIM images have demonstrated that a difference can be seen in the average lifetime of nanoprobe, which is dependent on the expression of target mRNA within cell lines. However, a limitation of this work is the small sample size of images for each condition. FLIM is a complex technique that took significant effort to optimise for the imaging of GNRs. Parameters such as the laser power and pinhole size were tested and optimised, as if the laser power was too high the GNRs heated up rapidly which resulted in the destruction of the cell of interest. The image cycle number was also optimised, to produce lifetime images that contained sufficient photon counts to provide accurate lifetime analysis. If they cycle count was too low there was not enough counts to produce a decay fit, whereas if the cycle count was too high the cells were at risk of destruction through GNR heating. Furthermore, the focusing within a cell was another parameter that was optimised. No fluorescent dye was used to identify the cell membrane or nucleus, as the only fluorescent signal that we wanted to measure within each cell was the nanoprobe. Therefore, it took time and experience to determine whether an image was

accurately focused within a cell, to measure the nanoprobe fluorescence intracellularly, and not focused above or below a cell of interest.

Now the FLIM sample preparation and imaging process has been optimised, future studies should be able to more rapidly prepare and image cells incubated with nanoprobe, which will allow different cell lines to be investigated. This will result in a larger data set which can be used to investigate the fluorescence lifetime differences of the nanoprobe in target mRNA expressing cells as compared to cells that do not express target mRNA.

6.4. Conclusions

The nano-bio interface is a complex process and has only recently been investigated; however, it is clear that nanoparticles can and do enter cells. The fate of these particles once inside the cell is less clear but these fluorescence intensity and fluorescence lifetime imaging results strongly support that the nanoprobe can come into contact with the target mRNA. This could be due to nanoparticle release from endosomes or fusion of the endosome with mRNA carrying vesicles.

Fluorescent confocal imaging and fluorescent lifetime imaging have been performed on fluorescent gold nanoprobe within cells. The fluorescence intensity of the nanoprobe in fluorescence confocal imaging was shown to be over 3-fold stronger in target mRNA expressing cancer cells than in non-cancer cells. The fluorescence intensity was also shown to be dependent on the focusing of the z position, as nanoprobe are found at different depths throughout cells. Therefore, for fluorescence sensing of gold nanoprobe, fluorescence intensity imaging provides complementary information to that of flow cytometry. Flow cytometry provides a more sensitive and robust method of detecting the fluorescence of nanoprobe and discriminating cancer cells from non-cancer cells, while fluorescence imaging provides information on the intracellular location of nanoprobe, as this can measure the total cell fluorescence and does not depend on the location of the nanoprobe.

It has also been demonstrated that FLIM provides a robust imaging technique to image GNRs within cells. The very short lifetime of GNRs, which is less than 100 ps, makes them a good contrast agent to biological dyes and cell autofluorescence, which typically display longer lifetimes between 1 and 3 ns. These results demonstrate that FLIM can distinguish the fluorescence lifetime of cy5 within the nanoprobe design can be used to distinguish cells that express a mRNA cancer biomarker based on the quenched state of the nanoprobe.

FLIM of C-MYC nanoprobe has demonstrated that in cell lines that have low expression of C-MYC mRNA, the fluorescent lifetime of cy5 is quenched due to the closed state of hpDNA. In cell lines that over express C-MYC the fluorescence lifetime of cy5 is restored, due to the opening of the hpDNA. When both cell lines were incubated with nanoprobe that had open hpDNA due to pre-incubation with a complimentary target, the lifetime of cy5 was restored and identical. This supports the theory that the lifetime of the nanoprobe is directly related to the hybridisation status of the hpDNA, with cy5 being quenched in cells with low target mRNA expression and unquenched in cells with high target mRNA expression. This makes FLIM a sensitive imaging technique to detect mRNA expression at the single cell level.

Furthermore, nanoprobe for RNA imaging provides advantages over previously reported imaging techniques, as nanorods can be uptaken by cells without the use of a transfection agent and the fluorescence intensity and fluorescence lifetime have been shown to be dependent on the expression of target mRNA, with reduced fluorescence intensity and reduced fluorescence lifetime in cells with low target mRNA expression that is recovered in cells with high target mRNA expression.

Although FLIM has been used to image GNRs before as contrast and fluorescence quenching agents, this is the first investigation that has utilised FLIM for investigating nanoprobe for sensing mRNA expression based on fluorescence lifetimes. FLIM provides several advantages over fluorescence intensity imaging, including high spatial resolution, low photobleaching due to NIR excitation, the

signal is independent from the fluorophore concentration, but it is dependent on the local environment, which allows the investigation into molecular interactions, such as FRET. In this investigation, it has been shown that the fluorescence lifetime of cy5 is directly related to the hybridisation status of the hpDNA of the nanoprobe. Therefore, FLIM provides the opportunity to use the fluorescence lifetimes of fluorophores within the nanoprobe design as an imaging technique for intracellular sensing of mRNAs.

7. Investigating the clinical potential of nanoprobes in liquid biopsy

7.1. Introduction

The ability to detect cancer mRNA biomarkers in liquid biopsies offers a non-invasive method for early disease diagnostics, predictive factors to guide appropriate treatment paths and disease progression monitoring. The presence and stability of mRNAs in liquid biopsies has been demonstrated, both as cell free circulating mRNA and mRNA within CTCs. CTCs are cells that are shed from a tumour and are carried around the circulation. CTCs can provide important prognostic information, as they carry DNA, RNA and protein biomarkers from the original tumour. Tumour related mRNA have been widely studied as biomarkers in liquid biopsies and tumour mRNA expression levels have been shown to be correlated with tumour burden (207). However, the frequency of CTCs has been reported to often be less than 1 per ml of blood, therefore the isolation and measurement of CTCs and mRNA biomarkers has been challenging.

The detection of blood-based cancer biomarkers has gained interest for a variety of different cancer types. Breast cancer is the most common malignancy and most common cause of cancer mortality in women. An American Cancer Society report stated that in 2016 breast cancer accounted for 29% of all new cancer diagnoses in women and is the leading cancer-related cause of death in women aged from 20 to 59 years (208). CTCs are detectable in more than 65% of patients with metastatic breast cancer, with over 5 CTC per 7.5 ml of blood (209), while early stage breast

cancer patients have been reported to have >1 CTCs per 7.5 ml of blood (210) and are reported to be detectable in around 25% of patients with localised breast cancer (211). As well as CTCs for cancer diagnostics, the detection of circulating mRNA within exosomes have also been explored for detecting cancer biomarkers. In breast cancer, studies have investigated the detection of microRNAs in exosomes to detect the cancer BrCa genes (212).

Current mechanisms of CTC capture and measurements include the use of magnetic beads coated in EpCAM antibodies, to capture CTCs that express this epithelial marker (213). However, this process limits the capture of CTCs to EpCAM expressing cells and due to the heterogeneity of tumours, some CTCs do not express EpCAM and this subpopulation will evade capture, resulting in a loss of data. Furthermore, this process can only deal with small sample volumes, processing 8 ml of whole blood in 2 hours, limiting the use of this technique for routine clinical use. A recent advancement on this method for isolating CTCs from whole blood is a process of flowing blood over an EpCAM antibody coated surface (214). This allows the processing of larger blood sample volumes, however, still remains the issue of not capturing CTCs that do not express or have low expression of EpCAM.

A recent method has been developed to combine CTC capture by size filtration with capture onto a glass slide for immunocytochemical staining to investigate morphological features and biomarker expressions of CTCs (215). However, this process is dependent on capture based on cell size which is not specific for CTCs and results in the unspecific capture of non-CTCs. Furthermore, this process then requires for CTCs to go through a cell staining process, during which CTCs could be lost or degraded.

Another method for the detection of CTCs are nanoflares, which have previously been discussed, and have been utilised for the detection of mRNA within CTCs. The nanoflare consists of a spherical gold nanoparticle functionalised with a monolayer of linear single stranded DNA. The DNA strand is hybridised to a shorter reporter recognition strand, that is complementary to the target mRNA. This reporter strand

contains a fluorophore that is quenched due to its proximity to the gold surface. When the target mRNA binds to the recognition sequence, the reporter strand is displaced from the nanoparticle, releasing the fluorophore, removing the fluorescence quenching. The fluorophore release results in a “flare” fluorescence signal. In this report, human blood was spiked with known concentrations of cancer cells, from the breast cancer cell line MDA-MB-231. The average recovery of cancer cells for concentrations between 10^2 and 10^6 cells per ml was 64%, with the ability to detect cancer cells down to 100 cells per ml (70). Therefore, it has been shown to be possible to detect circulating cancer cells using a fluorescent nanoparticle targeting cancer mRNA biomarkers.

There is a need to develop an improved method for the rapid, sensitive and accurate detection of CTCs and circulating mRNA biomarkers from liquid biopsies. In this study, the clinical potential for nanoprobe to be used in a screening test to detect cancer mRNA biomarkers in liquid biopsies was investigated, using pleural effusions and blood samples from cancer patients.

Pleural effusions, sometimes referred to as “water on the lungs”, is a build-up of excess fluid between the layers of the pleura outside the lungs. The presence of pleural effusion can be a sign of cancer, however it can exist in a benign form in response to infection or inflammation of the pleural tissue. When a malignant pleural effusion is present, this fluid contains cancer cells and this is a sign that the cancer has spread and is at an advanced stage (216). The most common cancers that are associated with malignant pleural effusions are lung cancer for men and breast cancer for women, which account for around 65% of all cases (217).

Pleural effusion fluid is removed by thoracentesis, which is a process that inserts a needle into the chest cavity to remove the fluid. Metastatic pleural effusions have been found to be a rich source of circulating tumour cells. In a clinical study of CTC isolation from pleural effusions the median number of cells found in metastatic patients was 526 per ml, with the highest number found in an individual patient exceeding 400,000 cells per ml. The median number of epithelial cells in patients

with benign pleural effusion was 6 (218). Another study looking to immunophenotype pleural effusion cells found that the median number of cells per ml in metastatic patients was 2121, compared to 12.5 in pleural effusions from non-cancer patients (219). Therefore, pleural effusion samples from metastatic patients contain an enriched sample of CTCs and provide an ideal liquid biopsy for testing the ability of nanoprobe to detect mRNA biomarkers within CTCs.

In this investigation, pleural effusion and whole blood liquid biopsies were collected from cancer patients and the ability for nanoprobe to detect cancer mRNA biomarkers was investigated through flow cytometry. This investigation resulted in positive preliminary data that the nanoprobe can detect circulating mRNA biomarkers in clinical liquid biopsies.

7.2. Sample preparation

7.2.1. Materials

Red cell lysis buffer (11814389001) was purchased from Sigma- Aldrich.

7.2.2. Clinical sample preparation and measurement by flow cytometry

Pleural effusion or blood samples were collected with consent from patients in the Shantou Tumour Hospital. Blood samples were treated with Red Blood Cell Lysis Buffer (Sigma) to remove red blood cells. 2 ml of room temperature lysis buffer was added to 1 ml of whole blood and the sample was mixed for 10 minutes. Samples were centrifuged at 500 x g for 5 minutes. The supernatant containing lysed red blood cells was removed and the pellet were resuspended in cell media. Each sample was prepared in duplicate; one sample was left untreated as a control and the other sample was treated with 0.1 nM of C-MYC or SOX2 nanoprobe. The samples were incubated at 37°C with mixing for 4 hours. Samples were then filtered through a 40 nm cell strainer to remove cell clumps.

Each sample was measured on a BD FACS Aria II flow cytometer. Cy5 was excited with a 633 nm laser, and the emission was collected with a 660/20 filter. 1 million

events were measured for each sample. The control sample was measured first, as this population was used to set a threshold gate for fluorescence. The nanoprobe treated sample was then measured and cells above the control threshold fluorescence were considered to be nanoprobe positive and express the C-MYC biomarker.

7.3. Results

7.3.1. Investigating the clinical applications of C-MYC nanoprobe in pleural effusion liquid biopsies

Pleural effusion samples were taken from 16 patients metastatic cancer patients and from 1 patient that had a benign pleural effusion and was cancer negative. Of these patients, 10 were breast cancer, 4 were lung cancer, 1 was endometrial cancer, 1 was unclassified and 1 participant was cancer negative.

For each patient sample, 1 ml of liquid biopsy was collected and was left untreated, as a control and 1 ml was incubated with 0.1 nM of C-MYC nanoprobe. The samples were incubated at 37°C for 4 hours, before being analysed for fluorescence by flow cytometry. For each patient sample, the untreated sample was measured first, followed by the analysis of the the nanoprobe treated sample. 1 million events were recorded for each control and nanoprobe treated sample. The fluorescence of cy5 within the nanoprobe was excited at 633 nm and the emission was collected through a 660/20 filter.

Flow cytometry analysis was performed using a histogram of fluorescence intensity versus count for control and nanoprobe treated pleural effusion samples. For each histogram graph, a gate was applied in the control sample, from the fluorescence intensity 0 and higher (gate A). The same gate was applied to the nanoprobe treated sample (gate B) and if a fluorescent peak was present, this could be visualised.

Analysis was also performed on the dot plot graph of fluorescence intensity versus side scatter. In each dot plot graph, every dot represents a cell or particle that has

been detected by the flow cytometer. For each dot plot analysis, a fluorescent gate was fitted closely around the control sample population (gate A) and the same gate was applied to the nanoprobe treated sample (gate B) to identify the difference in the fluorescence population. Fluorescence events that are present in gate B were considered to be a signal from the nanoprobe in association with C-MYC cell free ctRNA or C-MYC mRNA within CTCs.

Table 9 displays the dot plot analysis results for the percentage of fluorescence events observed in each pleural effusion sample after nanoprobe incubation.

Sample number	Cancer type	Fluorescent events after nanoprobe incubation (%)	Background (%)
1	Breast	1.39	0
2	Breast	0.16	0
3	Breast	3.15	0
4	Endometrial	0.6	0.01
5	Breast	0.5	0.0001
6	No cancer	0	0
7	Breast	1.44	0.0009
8	Breast	1.42	0.0022
9	Breast	0.17	0.0001
10	Breast	3.21	0.0015
11	Breast	0.95	0.0012
12	Unknown	1.08	0.0014
13	Lung	0.1	0.0017
14	Lung	0.18	0.0013
15	Lung	0.76	0.0006
16	Breast	1.92	0.0014
17	Lung	0.06	0.0014

Table 9. The percentage of fluorescence events detected by flow cytometry in pleural effusion patient samples following C-MYC nanoprobe incubation.

Of the 16 cancer positive samples, the nanoprobe treated sample displayed a variety of fluorescence positive populations, with a range from 0.06% to 3.21% and an average fluorescence population of 1.06%. The background value, which was taken as the fluorescent events in the control sample, also varied for each sample, with a background range from 0% to 0.01%. The limit of detection is considered to be 3x the value of the background, therefore the all of the pleural effusion samples incubated with C-MYC nanoprobe displayed a fluorescence population that is above the detection limit. Figure 49 shows the percentage distribution of the fluorescent population across all of the pleural effusion cancer positive samples. The highest proportion of fluorescence was seen at the 0.11-0.5% and the 1.1-1.5% values, with 25% (n=4) of samples showing a fluorescence population in each of these ranges after nanoprobe incubation.

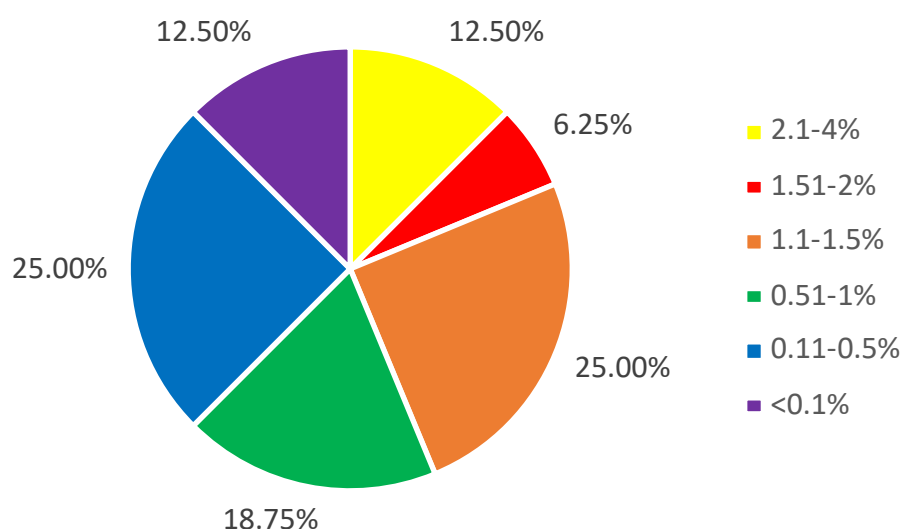


Figure 49. The percentage of fluorescence events detected in pleural effusion samples from cancer patients following nanoprobe incubation.

Pleural effusion samples from metastatic cancer patients were incubated with c-myc nanoprobes and analysed by flow cytometry. The fluorescence population was gated on a dot plot of fluorescence intensity versus side scatter using an untreated control sample as a reference. The percentage of fluorescence events were split into categories, using the legend above. The percentage of samples in each range have been plotted in a pie chart. The highest proportion of fluorescence was observed in the 1-1.5% range, with 25% of samples displaying this range of fluorescence events after nanoprobe incubation. The average fluorescence population observed after nanoprobe incubation was 1.06%.

The histograms and dot plot graphs of three different pleural effusion samples have been displayed. Sample 6 is the patient that was cancer negative. In the histogram analysis of this sample, there is no clear visual difference in the fluorescence intensity between the control and nanoprobe treated sample (Figure 50 a&b). Furthermore, when both histograms are gated about fluorescence intensity of 0, both samples display the same level of fluorescence background, with the control sample displaying 6,767 events and the nanoprobe sample displaying 6,811 events

(Figure 50 c&d). In the dot plot analysis for the control sample, when the cell population is excluded, gate A contains 0 events while in the nanoprobe treated sample gate B contains a very low background of 18 events (Figure 50 e&f). This represents a very small proportion of fluorescence background of 0.0018% of the total sample population. This demonstrates that the nanoprobe does not fluorescence when added to a patient liquid biopsy in the absence of cancer cells or cancer biomarkers.

Sample 8 was a pleural effusion sample from a metastatic breast cancer patient. In the histogram analysis, a fluorescence population can be visualised, as a fluorescence peak is present in the C-MYC nanoprobe treated sample that is not present in the control sample (Figure 51 a&b). When gate A was applied to the control sample histogram to detect events above 0, a background fluorescence from the control sample can be observed with 202 events recorded. When the same gate was applied to the nanoprobe treated sample, the events in this gate increased to 8,176, which represents 0.82% of the total sample population (Figure 51 c&d). This value of 0.82% represents the total fluorescent cell population within the nanoprobe treated sample but this value contains an background of 202 events, or 0.02%, that were detectable in the control sample. In the dot plot analysis of fluorescence intensity versus side scatter a gate was applied to exclude the control sample population, and this contains a background of 22 fluorescent events, giving a background value of 0.0022%. The same gate was applied to the nanoprobe treated sample and this gate B displayed a fluorescence population of 15,201 events, which represents 1.52% of the total sample population (Figure 51 e&f).

Sample 13 was a pleural effusion sample from a metastatic lung cancer patient. In the histogram analysis, the nanoprobe-incubated sample did not display an extra distinct fluorescence peak, however there was a visible shift in the tail of the histogram (Figure 52 a&b). When the histogram was gated to fluorescence intensity above 0, a background of 135 events was observed in the control sample, giving a background value of 0.0135%. When the same gate was applied to the nanoprobe treated sample, 2,205 events were detected, giving a fluorescent population of

0.22% (Figure 52 c&d). In the dot plot analysis of fluorescence intensity versus side scatter, gate A was applied around the control sample to exclude nearly all of the control sample population and resulted in 17 background fluorescent events, giving an background value of 0.0017%. The same gate was applied to the nanoprobe treated sample and this gate B displayed a fluorescence population of 2,126 events, which represents 0.21% of the total sample population (Figure 52 e&f).

These figures show the analysis of three pleural effusion samples, one non-cancer sample, one breast cancer sample and one lung cancer sample. Both the histogram analysis and the dot plot analysis techniques demonstrate that there is a fluorescence population in the pleural effusion sample from a metastatic cancer patient that was incubated with the C-MYC nanoprobe, that was not present in the control sample. Furthermore, the analysis shows that in a non-cancer patient, there was no fluorescence from the nanoprobe, demonstrating the specific activation in response to cancer signatures.

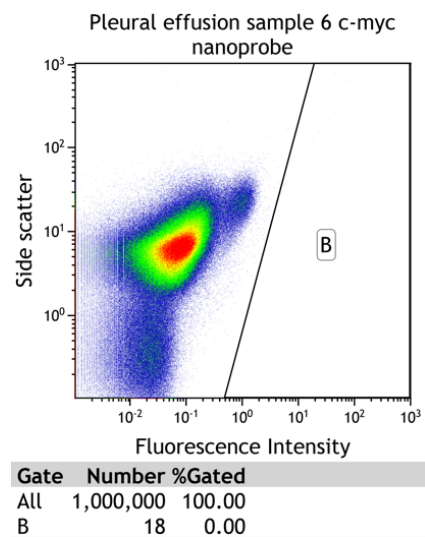
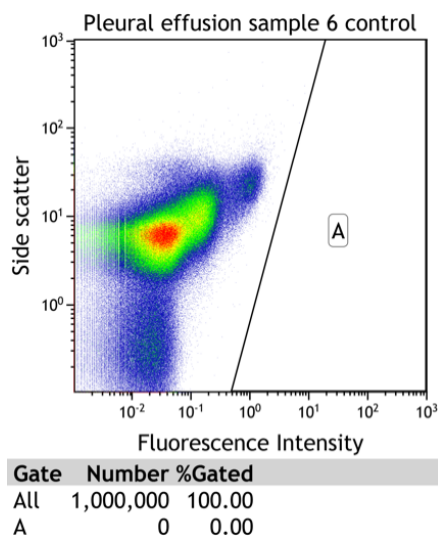
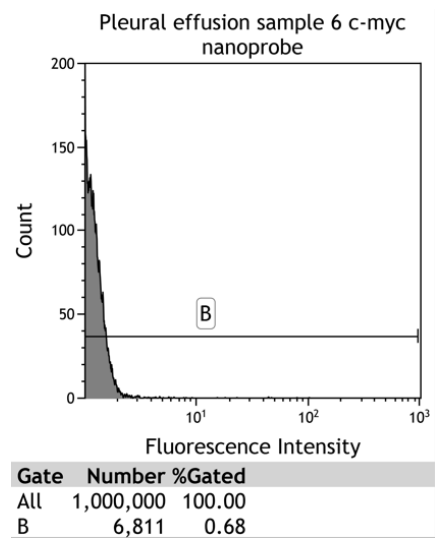
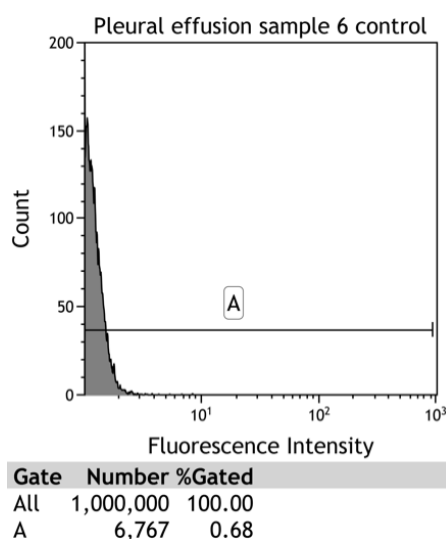
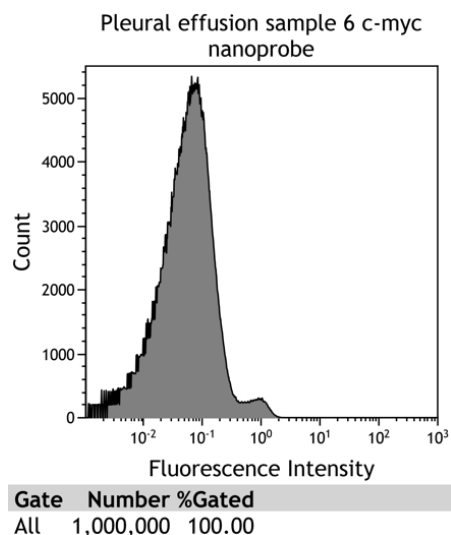
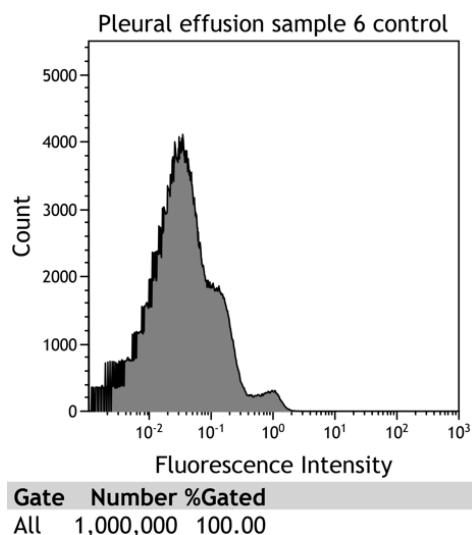


Figure 50. Flow cytometry analysis of a benign pleural effusion liquid biopsy following incubation with a c-myc nanoprobe.

Flow cytometry analysis of a non-cancer pleural effusion sample through a histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Histogram analysis of fluorescence events above 0 intensity for c) control sample and d) nanoprobe treated sample. Analysis was also performed on a dot plot of fluorescence intensity versus side scatter. e) Gate A was applied to exclude all of the control sample. f) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. In a non-cancer pleural effusion sample, no difference in fluorescence events can be observed. This suggests that the nanoprobe has not been activated in a non-cancer sample.

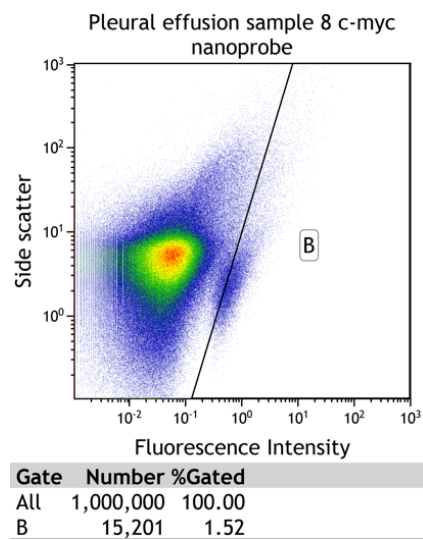
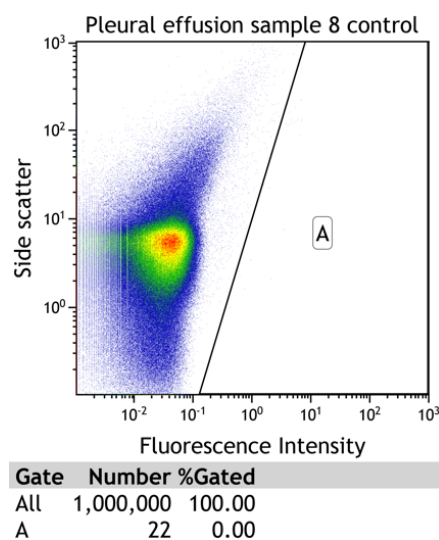
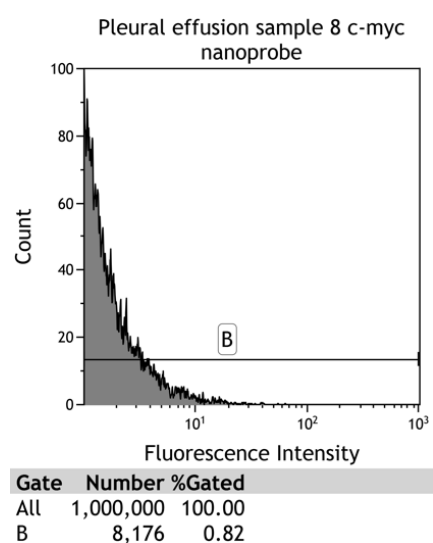
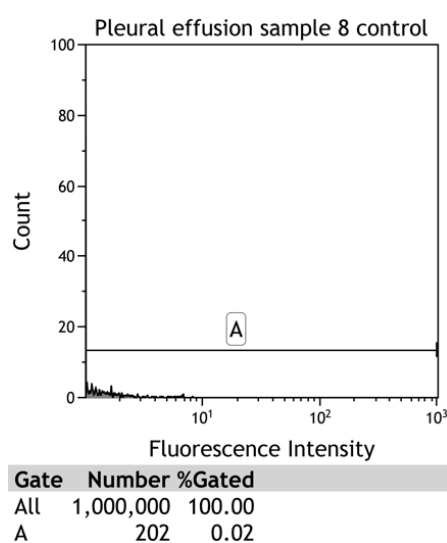
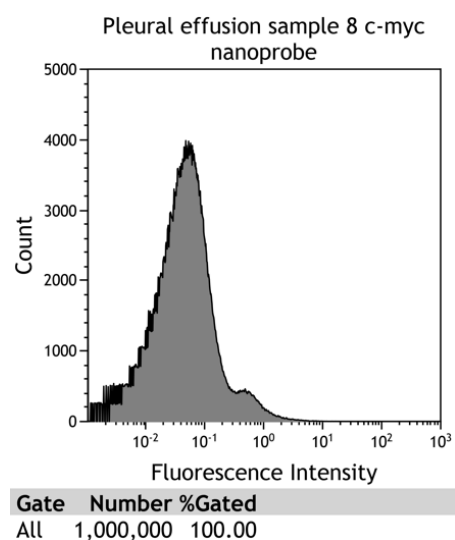
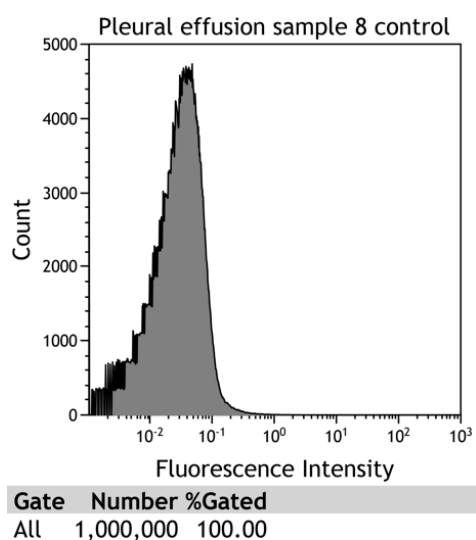


Figure 51. Flow cytometry analysis of a metastatic breast cancer pleural effusion liquid biopsy following incubation with a c-myc nanoprobe.

Flow cytometry analysis of a metastatic breast cancer pleural effusion sample through a histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Histogram analysis was performed for fluorescence events above 0 intensity for c) control sample and d) nanoprobe treated sample. Analysis was also performed on a dot plot of fluorescence intensity versus side scatter e) gate A was applied to exclude all of the control sample. f) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. In this breast cancer pleural effusion sample, a difference in fluorescence events after nanoprobe incubation can be observed after histogram analysis of 0.82% and dot plot analysis of 1.52%. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

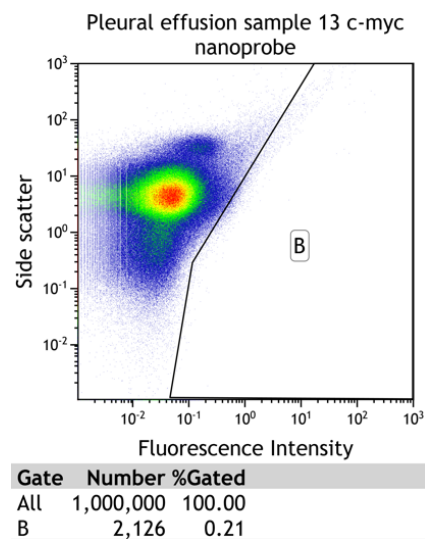
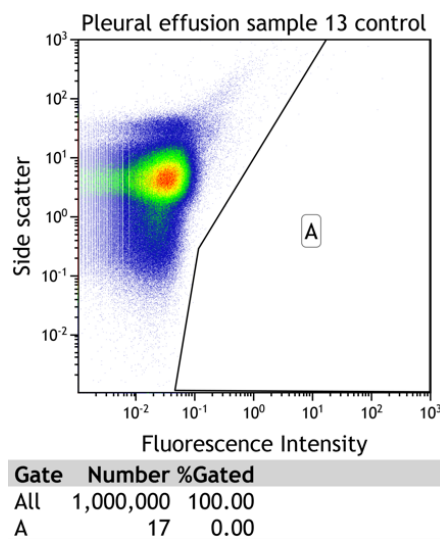
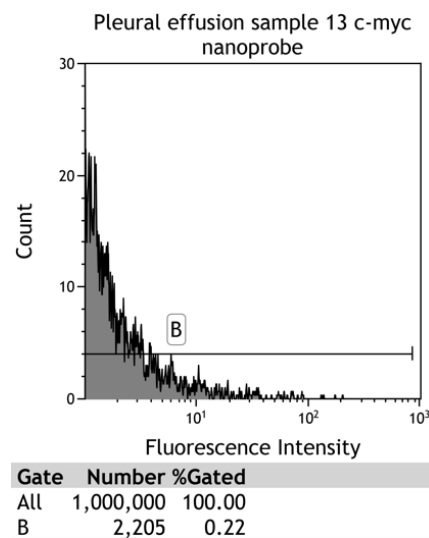
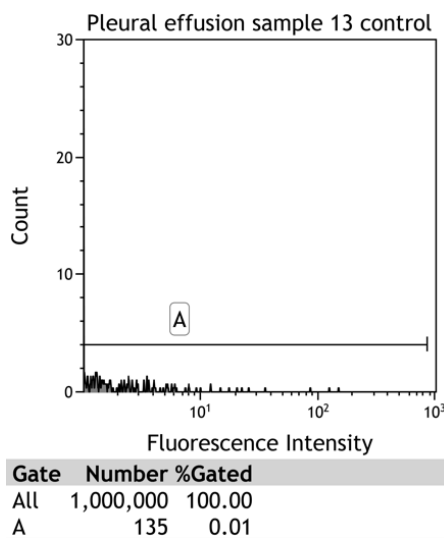
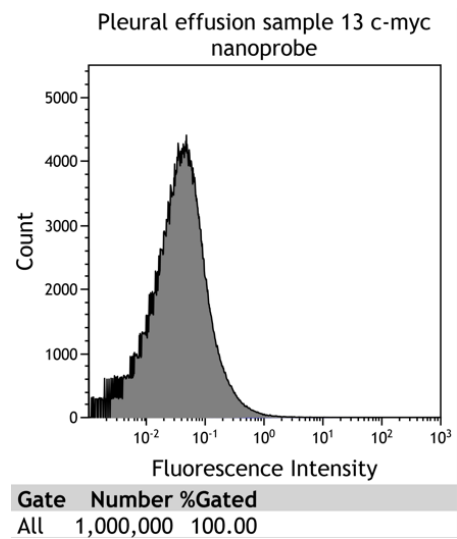
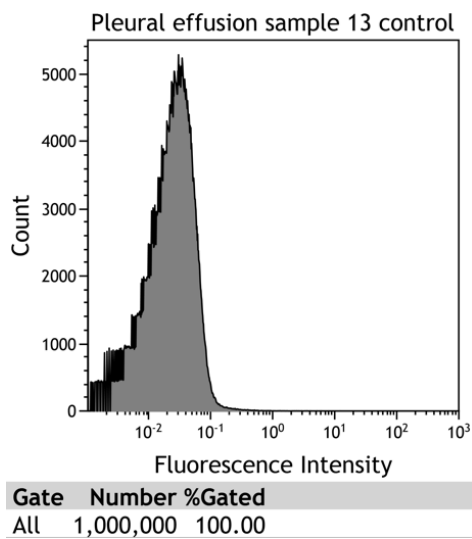


Figure 52. Flow cytometry analysis of a metastatic lung cancer pleural effusion liquid biopsy following incubation with a c-myc nanoprobe.

Flow cytometry analysis of a metastatic lung cancer pleural effusion sample through a histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Histogram analysis was performed for fluorescence events above 0 intensity for c) control sample and d) nanoprobe treated sample. Analysis was also performed on a dot plot of fluorescence intensity versus side scatter e) gate A was applied to exclude all of the control sample. f) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. In this lung cancer pleural effusion sample, a difference in fluorescence events after nanoprobe incubation can be observed after histogram analysis, of 0.22% and dot plot analysis of 0.21%. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

7.3.2. Investigating the clinical applications of SOX2 nanoprobe in pleural effusion liquid biopsies

The same pleural effusion samples that were analysed with C-MYC nanoprobe were also incubated and analysed with SOX2 nanoprobe, when there was enough sample volume to allow a duplicate nanoprobe incubation. In total, 7 pleural effusion samples were incubated with SOX2 nanoprobe. These samples consisted of 2 breast cancer, 4 lung cancer and 1 unclassified cancer.

For each sample, a control untreated pleural effusion sample was prepared alongside a nanoprobe treated sample. For nanoprobe incubation, 0.1 nM of SOX2 nanoprobe was added to a 1 ml sample and incubated for 4 hours at 37°C before analysis by flow cytometry. Flow cytometry analysis was performed using a histogram of fluorescence intensity versus count for control and nanoprobe treated pleural effusion samples. For each histogram graph, a gate was applied in the control sample, from the fluorescence intensity 0 and higher (gate A). The same

gate was applied to the nanoprobe treated sample (gate B) and if a fluorescent peak was present, this could be visualised.

Analysis was also performed on the dot plot graph of fluorescence intensity versus side scatter. For each dot plot analysis, a fluorescent gate was fitted closely around the control sample population (gate A) and the same gate was applied to the nanoprobe treated sample (gate B) to identify the difference in the fluorescence population. In both methods of analysis, fluorescence events in gate B were considered to be a signal from the nanoprobe in association with SOX2 cell free ctRNA or SOX2 mRNA within CTCs. Table 10 displays the percentage of fluorescence events detected after SOX2 nanoprobe incubation with each pleural effusion sample.

Sample number	Cancer type	Fluorescent events after nanoprobe incubation (%)	Background (%)
1	breast	8.27	0
2	breast	3.5	0.0009
3	unknown	0.84	0.001
4	lung	0.1	0.0017
5	lung	2.63	0.0033
6	lung	0.55	0.0029
7	lung	2.81	0.0014

Table 10. The percentage of fluorescence events detected by flow cytometry in pleural effusion patient samples following SOX2 nanoprobe incubation.

Of the 7 cancer positive samples treated with SOX2 nanoprobe, all samples displayed a fluorescence population that was not present in the control sample.

The nanoprobe treated samples displayed a variety of fluorescence positive populations, with a range from 0.1% to 8.27% and an average fluorescence population of 2.65%. The background value also varied for each sample, with a background range from 0% to 0.0033%. The limit of detection is considered to be 3x the value of the background, therefore the all of the pleural effusion samples incubated with SOX2 nanoprobe displayed a fluorescence population that is above the detection limit.

The SOX2 incubated pleural effusion sample 2 was from a metastatic breast cancer patient. In the histograms of fluorescence intensity versus count, a clear difference can be visualised between the control and nanoprobe incubated sample, with an extra fluorescence intensity peak present following nanoprobe incubation (Figure 53 a&b). When the histograms were gated to events over 0 intensity, the control sample displays a background value of 267 events, leading to a background value of 0.0267% (Figure 53c). In the SOX2 nanoprobe sample, this value increased to over 19,000 fluorescence events, which represents 1.9% of the total sample population (Figure 53d). Analysis was also performed on a dot plot of fluorescence intensity versus side scatter. In the dot plot analysis, gate A was applied to exclude the control sample population, and this gate contains a background value of 9 fluorescence events, giving a background value of 0.0009% (Figure 53e). When the same gate (gate B) is applied to the nanoprobe treated sample, and this gate displayed over 32,000 events, representing 3.28% of the total population (Figure 53f).

The SOX2 incubated pleural effusion sample 3 is from a cancer patient whose cancer type was not provided. In the histograms of fluorescence intensity versus count, a clear difference can be visualised between the control and nanoprobe incubated sample, with an extra fluorescence intensity peak present following nanoprobe incubation (Figure 54a&b). When the histograms were gated to events over 0 intensity, the control sample displays a background value of 1,369 events, leading to a background value of 0.14% (Figure 54c). In the SOX2 nanoprobe sample, this value increased to over 43,000 fluorescence events, which represents

4.31% of the total sample population (Figure 54d). Analysis was also performed on a dot plot of fluorescence intensity versus side scatter. In the dot plot analysis, gate A was applied to exclude the control sample population, and this gate contains a background value of 10 fluorescence events, giving a background value of 0.001% (Figure 54e). When the same gate (gate B) is applied to the nanoprobe treated sample, and this gate displayed over 8,000 events, representing 8.4% of the total population (Figure 54f).

The SOX2 incubated pleural effusion sample 4 is from a metastatic lung cancer patient. In the histograms of fluorescence intensity versus count, there is not an obvious difference that can be visualised between the control and nanoprobe incubated sample, however there is an elongated tale on the histogram following nanoprobe incubation (Figure 55a&b). When the histograms were gated to events over 0 intensity, the control sample displays a background value of 130 events, leading to a background value of 0.013% (Figure 55c). In the SOX2 nanoprobe sample, this value increased to over 1,200 fluorescence events, which represents 0.12% of the total sample population (Figure 55d). Analysis was also performed on a dot plot of fluorescence intensity versus side scatter. In the dot plot analysis, gate A was applied to exclude the control sample population, and this gate contains a background value of 17 fluorescence events, giving a background value of 0.0017% (Figure 55e). When the same gate (gate B) is applied to the nanoprobe treated sample, and this gate displayed over 1,500 events, representing 0.15% of the total population (Figure 55f).

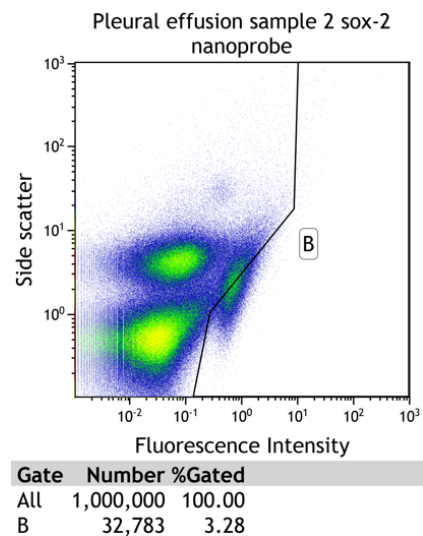
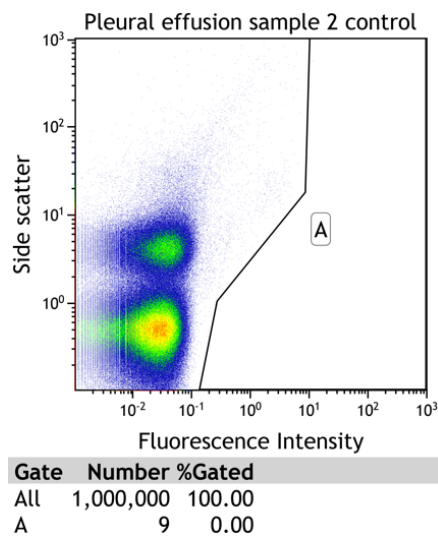
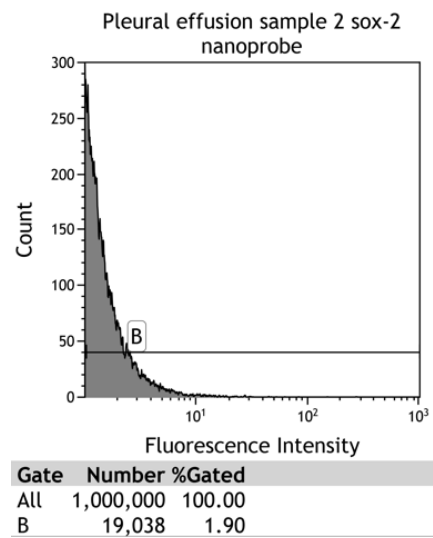
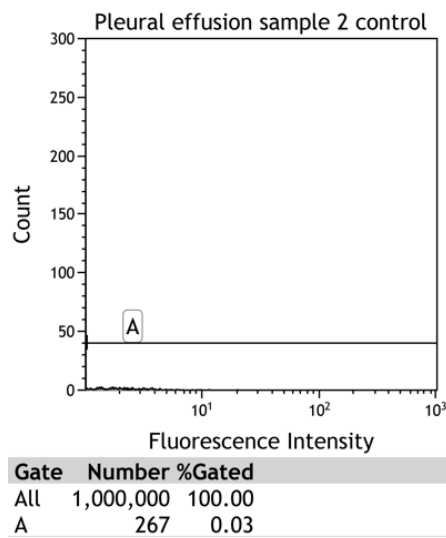
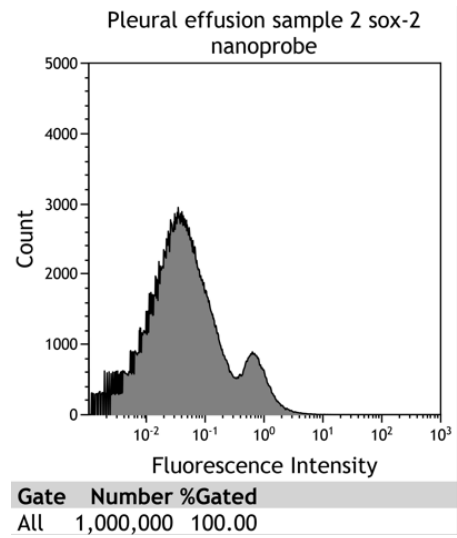
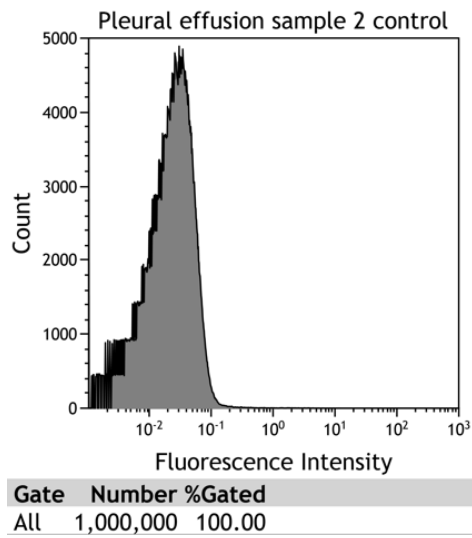


Figure 53. Flow cytometry analysis of a metastatic breast cancer pleural effusion liquid biopsy following incubation with a SOX2 nanoprobe.

Flow cytometry analysis of a metastatic breast cancer pleural effusion sample through a histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Histogram analysis was performed for fluorescence events above 0 intensity for c) control sample and d) nanoprobe treated sample. Analysis was also performed on a dot plot of fluorescence intensity versus side scatter e) gate A was applied to exclude all of the control sample. f) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. In this pleural effusion sample, a fluorescent population of 1.9% in the histogram analysis and 3.29% by dot plot analysis can be observed after nanoprobe incubation. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

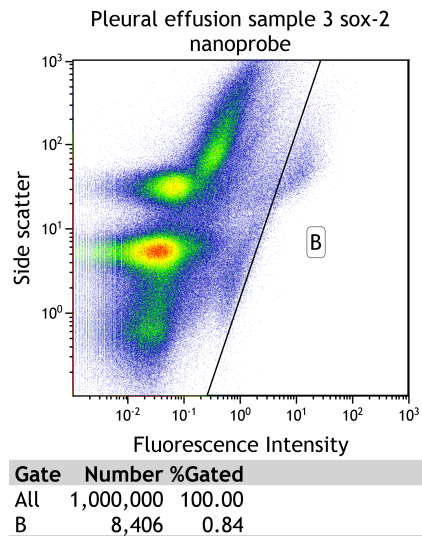
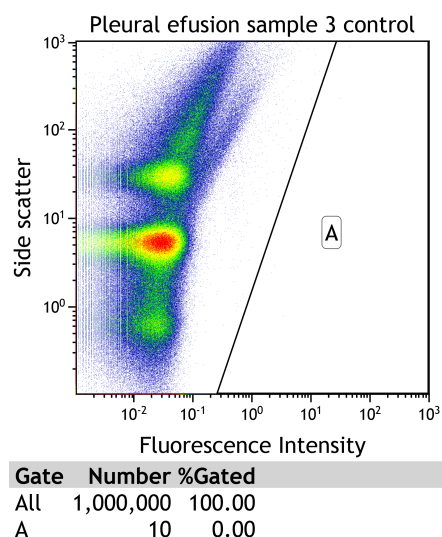
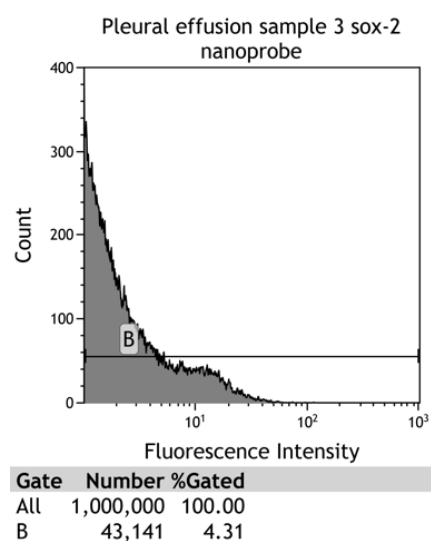
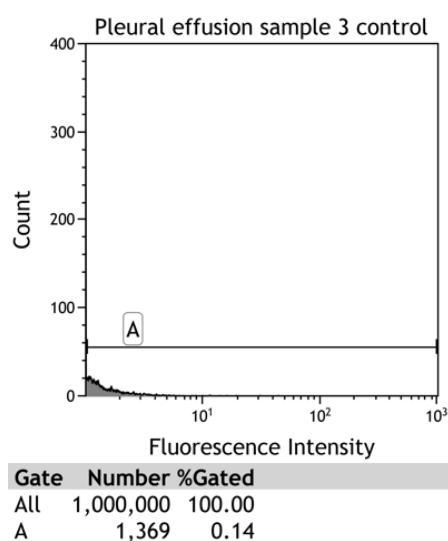
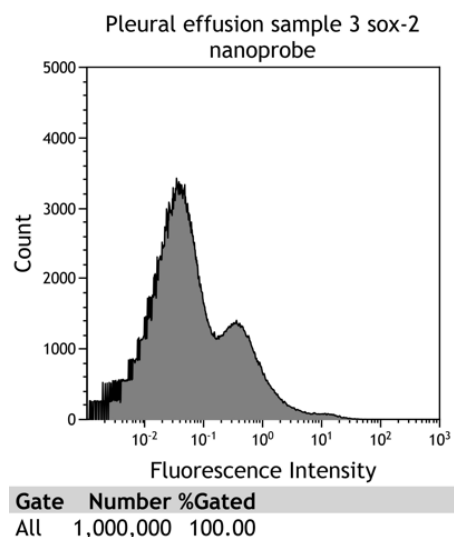
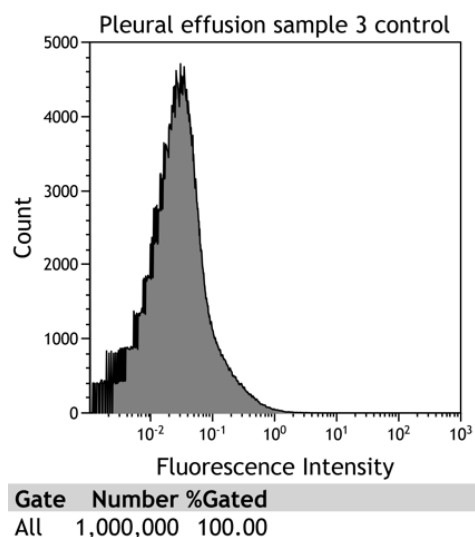


Figure 54. Flow cytometry analysis of a metastatic cancer pleural effusion liquid biopsy following incubation with a SOX2 nanoprobe.

Flow cytometry analysis of a metastatic breast cancer pleural effusion sample through a histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Histogram analysis was performed for fluorescence events above 0 intensity for c) control sample and d) nanoprobe treated sample. Analysis was also performed on a dot plot of fluorescence intensity versus side scatter e) gate A was applied to exclude all of the control sample. f) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. In this pleural effusion sample, a fluorescent population of 4.21% in the histogram analysis and 0.84% by dot plot analysis can be observed after nanoprobe incubation. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

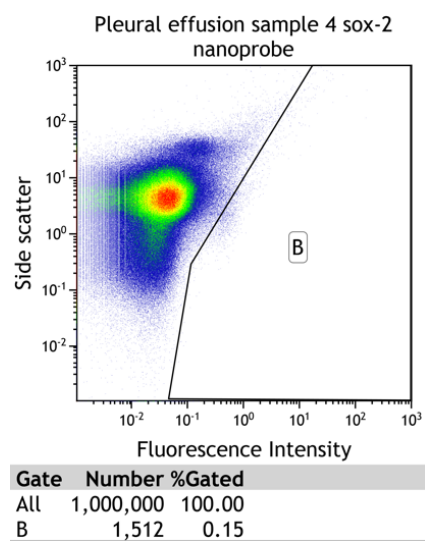
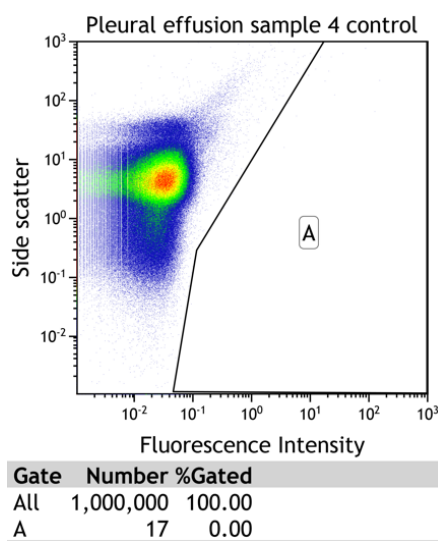
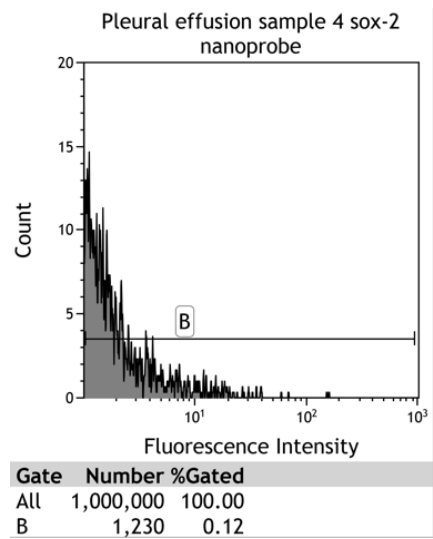
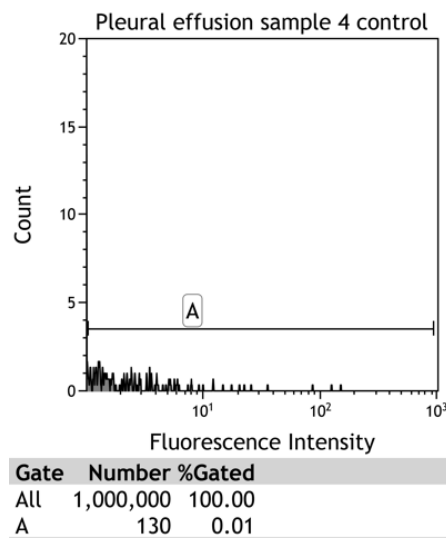
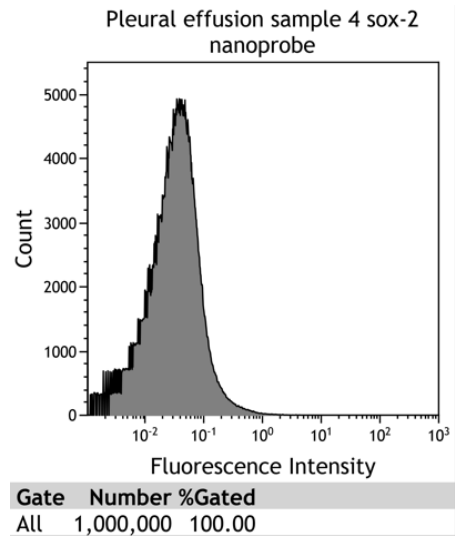
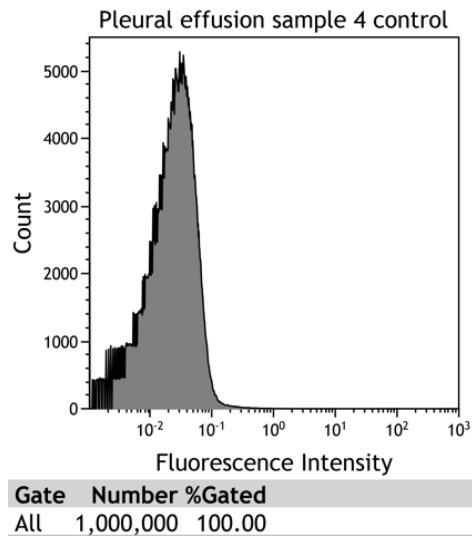


Figure 55. Flow cytometry analysis of a metastatic lung cancer pleural effusion liquid biopsy following incubation with a SOX2 nanoprobe.

Flow cytometry analysis of a lung cancer pleural effusion sample through a histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Histogram analysis was performed for fluorescence events above 0 intensity for c) control sample and d) nanoprobe treated sample. Analysis was also performed on a dot plot of fluorescence intensity versus side scatter e) gate A was applied to exclude all of the control sample. f) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. In this pleural effusion sample, a fluorescent population of 0.12% in the histogram analysis and 0.15% by dot plot analysis can be observed after nanoprobe incubation. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

Figures 53-55 show the analysis of 3 pleural effusion liquid biopsies from cancer patients that have been incubated with the SOX2 nanoprobe. Both the histogram analysis and the dot plot analysis techniques demonstrate that there is a fluorescence population in the pleural effusion sample from a metastatic cancer patient that was incubated with the SOX-2 nanoprobe, that was not present in the control sample. As can be seen in Table 10, each sample incubated with SOX2 nanoprobe showed a different proportion of fluorescence events.

For both C-MYC and SOX2 nanoprobe treated pleural effusion samples, the dot plot analysis is thought to be the most appropriate method for the analysis of the nanoprobe fluorescence. This is because each sample displayed differential background fluorescence and gating based on the dot plot graph allows a more accurate gating of the control sample population and more accurately defines the fluorescence population from the nanoprobe. However, gating the dot plot graph

does lead to some background signal, as there can be a small proportion of the control sample that are within the gate and lead to unspecific background signal.

A limitation of this work is the limited number of samples that were incubated with SOX-2 nanoprobe. Ideally, every sample would have been incubated with both C-MYC and SOX-2 nanoprobe. However, the limited sample volumes that were received for analysis meant that the majority of samples could only be analysed with one nanoprobe, and C-MYC was the priority for this investigation. Therefore, only 7 patient samples were incubated with both C-MYC and SOX-2 nanoprobe.

In order to investigate if the signal intensity from pleural effusion samples incubated with C-MYC nanoprobe correlated with the signal intensity from the same patient sample that was incubated with SOX-2 nanoprobe, a comparison of samples taken from the same patients was performed (Table 11). Out of all the patient samples, combined patient number 1 displayed the highest overall intensity for both C-MYC and SOX-2 nanoprobe. This suggests that this patient sample, which was breast cancer, displayed high expression of C-MYC and SOX-2 mRNA within CTCs or as cell free circulating biomarkers. Combined patient 3 (unknown cancer), 4 (lung) and 6 (lung) have similar fluorescence intensity values for both nanoprobe, suggesting these patients have expression of both biomarkers of interest. Combined patient 5 (lung) displayed low intensity with the C-MYC nanoprobe, which increased 14.6-fold with SOX-2 nanoprobe. This suggests that this patient may highly express the SOX-2 biomarker, in comparison with C-MYC.

It is not surprising that there is not a clear correlation of fluorescence intensity from the same sample using different nanoprobe, as the expression of C-MYC and SOX-2 biomarkers within a cancer cell are not co-dependant and do not both have to be present for a cancer to exist. With only 7 samples to compare C-MYC and SOX-2 expression, it is not possible to determine if a correlation occurs within a specific cancer subtype, where the expression of both C-MYC and SOX-2 may both contribute to the cancer's progression. A clinical trial investigating the expression of different biomarkers within different cancer subtypes, and the nanoprobe

fluorescence that is produced in these samples would be a valuable future investigation for the clinical potential of nanoprobe. To confirm the mRNA expression levels of each biomarker within each patient sample, further studies using PCR would be required to extract the true mRNA expression levels. These results highlight the need for multiplexed biomarkers, as no one biomarker can reliably detect the presence of cancer cells.

c-myc sample number	sox2 sample number	Combined patient number	c-myc signal	sox2 signal
3	1	1	3.15	8.27
11	2	2	0.95	3.5
12	3	3	1.08	0.84
13	4	4	0.21	0.1
14	5	5	0.18	2.63
15	6	6	0.76	0.55
17	7	7	0.06	2.81

Table 11. Comparing the fluorescent signal intensity of effusion samples from individual patients that were incubated with both C-MYC and SOX-2 nanoprobe.

7.3.3. Investigating the clinical applications of nanoprobe in blood sample liquid biopsies

Following the preliminary positive results from pleural effusion samples incubated with C-MYC nanoprobe, the potential of the same nanoprobe to detect cancer the C-MYC mRNA biomarker in blood samples was investigated. Blood samples were collected from metastatic cancer patients at the Shantou Tumour Hospital. A total of 19 blood samples were tested, of these patients there were 12 breast cancer, 2 oesophageal cancer, 1 lung cancer, 1 colorectal cancer, 1 cervical cancer, 1 cardiac cancer and 1 unrecorded cancer type.

For each blood sample, 1 ml was collected and was left untreated as a control and 1 ml was incubated with 0.1 nM of C-MYC nanoprobe. The difference between pleural effusion sample processing and blood sample processing was the use of red cell lysis buffer. Blood samples contain a high background of red blood cells, so to remove this from the sample a red cell lysis buffer was used before nanoprobe incubation. The samples were incubated at 37°C for 4 hours, before being analysed for fluorescence by flow cytometry. For each patient sample, the untreated sample was measured first and then the nanoprobe treated sample was measured. 1 million events were recorded for each control and nanoprobe treated sample. The fluorescence of cy5 within the nanoprobe was excited at 633 nm and the emission was collected through a 660/20 filter.

Flow cytometry analysis was performed on a dot plot graph of fluorescence intensity versus side scatter. For each sample analysis, a gate was fitted closely around the control sample population (gate A) and the same gate was applied to the nanoprobe treated sample (gate B). The difference in the fluorescence population in gate B, as compared to gate A was considered to be fluorescence from the nanoprobe. Table 12 displays the dot plot analysis results for the percentage of fluorescence events observed in each blood sample after nanoprobe incubation.

Sample number	Cancer type	Fluorescent events after nanoprobe incubation (%)	Background (%)
1	Unknown	0.93	0.0011
2	Oesophageal	0.15	0.0018
3	Breast	0.03	0.0025
4	Cardiac	0.03	0.0032
5	Lung	0.79	0.0042
6	Breast	0.74	0.0036
7	Breast	2.01	0.0029
8	Breast	0.07	0.003
9	Breast	0.39	0.0015
10	Breast	0.14	0.0022
11	Colorectal	0.14	0.0005
12	Breast	0.3	0.002
13	Breast	0.03	0.002
14	Breast	0.002	0.0021
15	Breast	0.07	0.0026
16	Breast	0.14	0.0016
17	Cervical	0.26	0.0014
18	Oesophageal	0.27	0.0028
19	Breast	0.03	0.0027

Table 12. The percentage of fluorescence events detected by flow cytometry in blood samples from cancer patients following nanoprobe incubation.

The dot plot fluorescence analysis displayed a variety of fluorescence populations after nanoprobe incubation, with a range from 0.002% to 2.01%, with an average fluorescence population of 0.34%. The background value also differed for each sample, with a background value range of 0.0005% to 0.0042%. The limit of

detection is considered to be 3x the value of the background, therefore the majority of samples displayed a fluorescence population that is above the detection limit. Sample 14 is the exception, where no difference was seen between the control and C-MYC nanoprobe treated sample. Figure 56 shows the percentage distribution of the fluorescent population across all of the cancer blood samples. The highest proportion of samples displaying fluorescence was observed between 0.01-0.1% range, with 36.84% (n=7) of samples demonstrating a fluorescence population in this range.

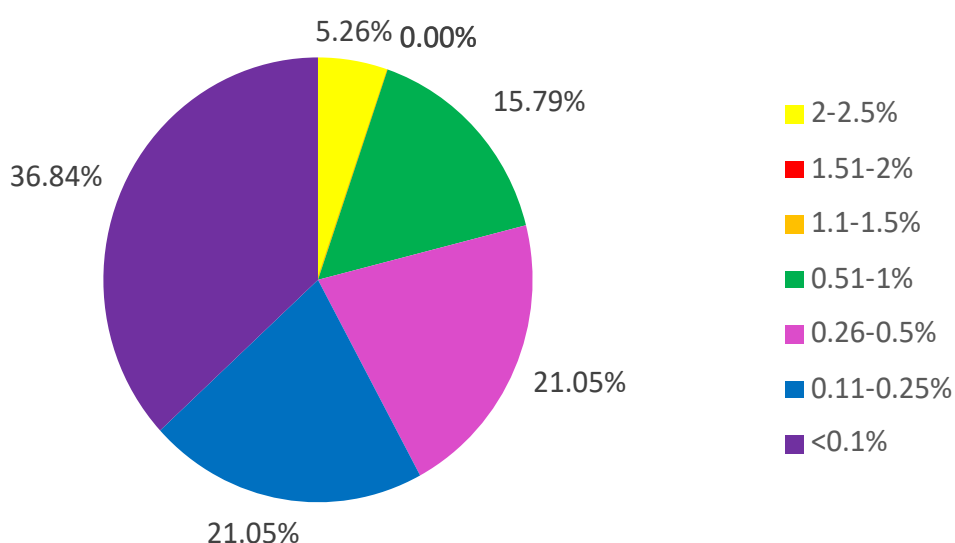


Figure 56. The percentage of fluorescence events detected in blood samples from cancer patients following nanoprobe incubation.

Blood samples from metastatic cancer patients were incubated with c-myc nanoprobes and analysed by flow cytometry. The fluorescence population was gated on a dot plot of fluorescence intensity versus side scatter using an untreated control sample as a reference. The percentage of fluorescence events detected in nanoprobe treated samples were split into ranges, using the legend above. The percentage of samples in each range have been plotted in a pie chart. The highest proportion of fluorescence was observed in the 0-0.1% range, with 36.84% of samples displaying this range of fluorescence events after nanoprobe incubation. The average fluorescence population observed after nanoprobe incubation was 0.34%.

The dot plot gate analysis for three different blood samples has been shown, to demonstrate the range of fluorescence events that were detected. Histogram analysis was not performed for blood samples, as they displayed a higher background fluorescence as compared to pleural effusion samples and there was not always a clear cut off to gate the samples.

Sample 6 was a blood sample from a breast cancer patient. In the histograms for this sample a distinct fluorescence peak could be observed in the nanoprobe treated sample that is not present in the control sample (Figure 57 a&b). In the dot plot analysis of fluorescence intensity versus side scatter gate A was applied around the control sample, to exclude nearly all of the control sample population. Gate A contained a very low background fluorescence of 36 events, which provides a background value of 0.0036%. The same gate was applied to the nanoprobe treated sample and this gate B displayed a fluorescence population of 7,400 events, which represents 0.74% of the total sample population (Figure 57 c&d). Furthermore, when comparing the dot plot graphs, a clear shift in the fluorescence intensity can be visualised within the nanoprobe treated sample. Therefore, the nanoprobe has exhibited a fluorescence signal in 0.74% of the sample population.

Sample 15 was a blood sample from a breast cancer patient. In the histogram of control and nanoprobe treated sample, both samples display a tale on the graph, but it appears that there is a more defined second fluorescence peak in the nanoprobe treated sample (Figure 58 a&b). When the gate A was applied to the control sample this excluded the majority of the control sample population and displayed a low fluorescence background of 26 events, which provides a background value of 0.0026%. When the same gate was applied to the nanoprobe treated sample this displayed 706 events, which represents 0.07% of the population (Figure 58 c&d). Similarly, in comparing the dot plot graphs a new population can be observed in the nanoprobe treated sample that display a higher fluorescence intensity than the events in the control sample.

Sample 17 was a blood sample from a cervical cancer patient. From the histograms of control and nanoprobe treated samples, there does not appear to be a clear difference in the fluorescence intensity (Figure 59 a&b). Gate A was applied to exclude the control sample population, and this resulted in a low fluorescence background of 14 events, giving a background value of 0.0014%. When the same gate was applied to the nanoprobe treated sample, a fluorescence population that was not present in the control sample was captured, of 2,563 events, which represents 0.26% of the total sample population (Figure 59 c&d).

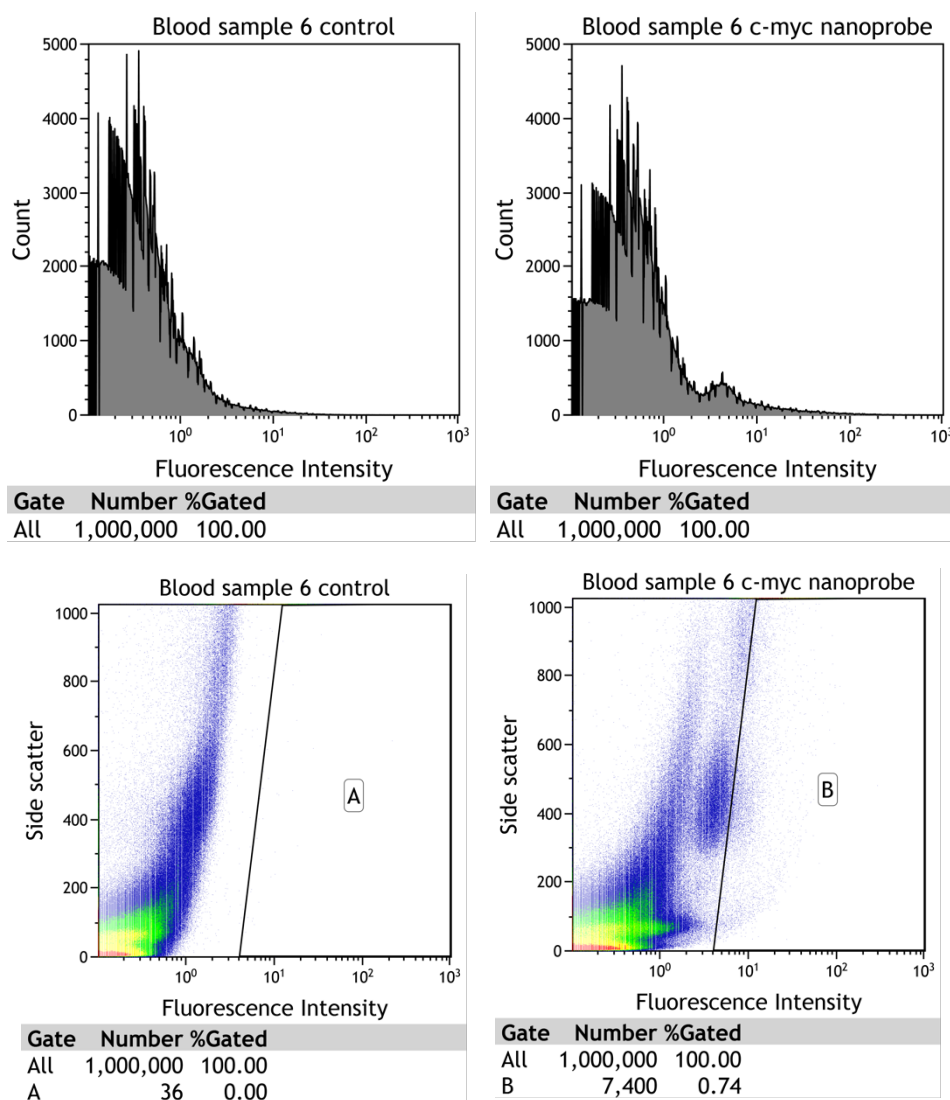


Figure 57. Flow cytometry analysis of a metastatic breast cancer blood sample liquid biopsy following incubation with a C-MYC nanoprobe.

Flow cytometry analysis of a blood sample from a metastatic breast cancer patient. A histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Analysis was performed on a dot plot of fluorescence intensity versus side scatter. c) Gate A was applied to exclude all of the control sample. d) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. A difference in fluorescence events after nanoprobe incubation of 0.74% can be observed. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

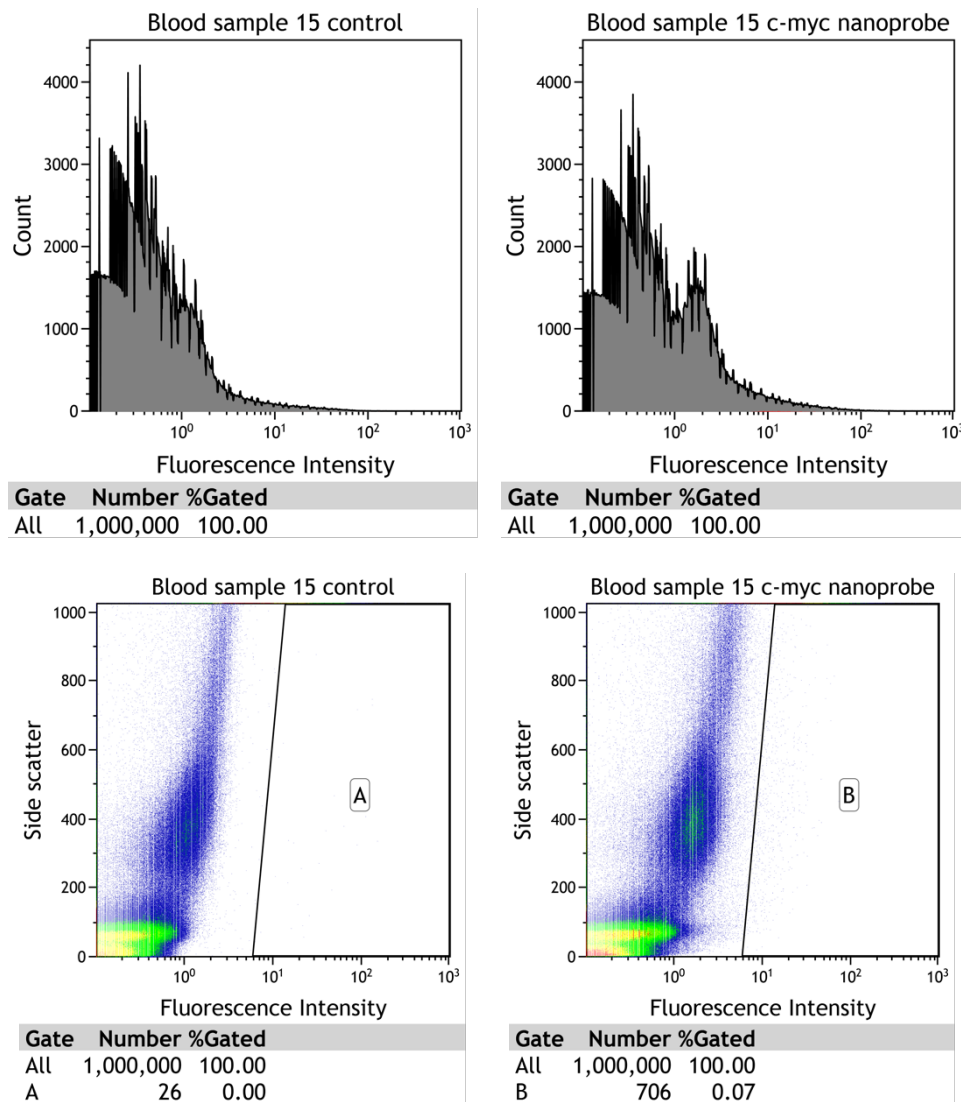


Figure 58. Flow cytometry analysis of a metastatic breast cancer blood sample liquid biopsy following incubation with a C-MYC nanoprobe.

Flow cytometry analysis of a blood sample from a metastatic breast cancer patient. A histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Analysis was performed on a dot plot of fluorescence intensity versus side scatter. c) Gate A was applied to exclude all of the control sample. d) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. A difference in fluorescence events after nanoprobe incubation of 0.07%. can be observed. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

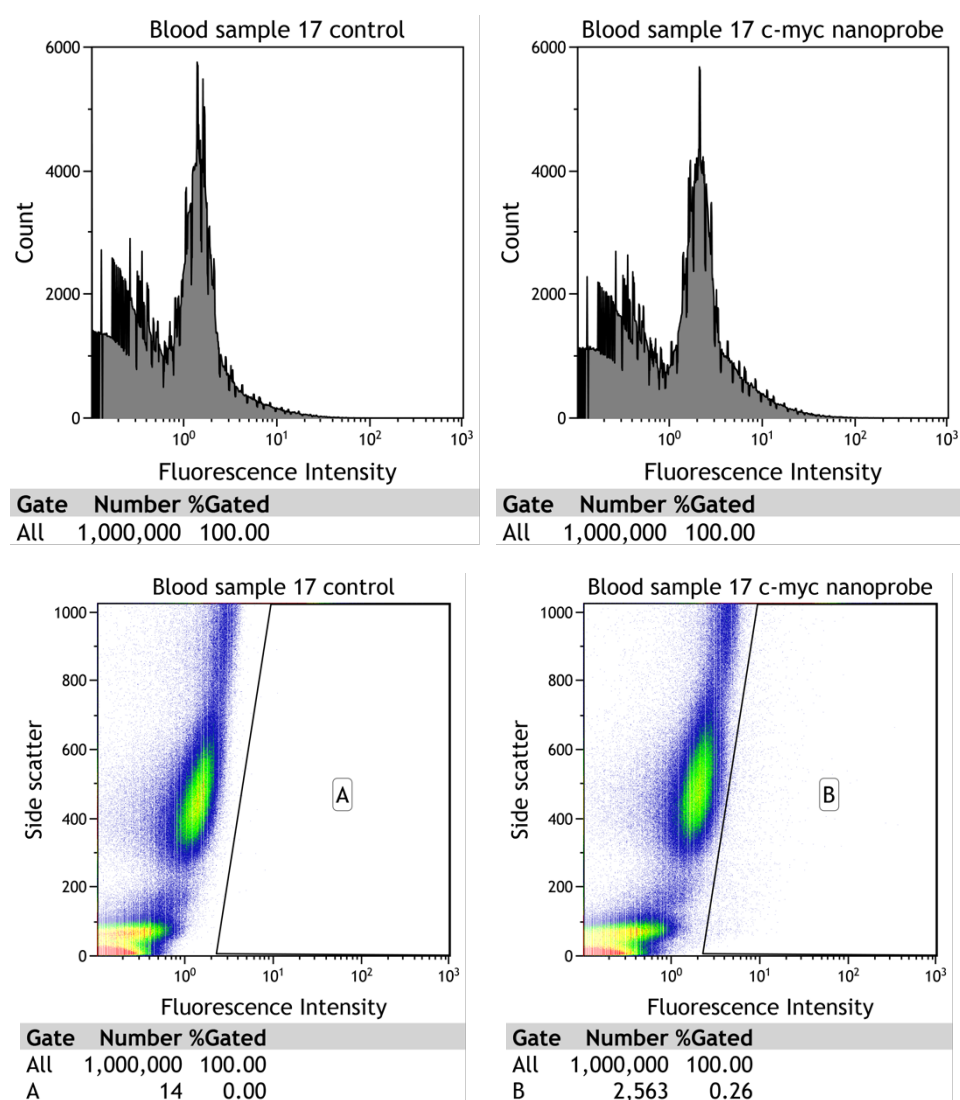


Figure 59. Flow cytometry analysis of a metastatic cervical cancer blood sample liquid biopsy following incubation with a C-MYC nanoprobe.

Flow cytometry analysis of a blood sample from a metastatic breast cancer patient. A histogram of fluorescence intensity versus count for a) untreated control sample b) nanoprobe treated sample. Analysis was performed on a dot plot of fluorescence intensity versus side scatter. c) Gate A was applied to exclude all of the control sample. d) The same gate was applied to the nanoprobe treated sample to observe any differences in fluorescence events. A difference in fluorescence events after nanoprobe incubation of 0.26% can be observed. This suggests that the nanoprobe has been activated in response to the cancer mRNA biomarker.

Finally, the results from flow cytometry analysis from all liquid biopsy samples have been combined in Figure 60. This shows box plot analysis to identify the range of fluorescence events detected by flow cytometry in pleural effusion samples incubated with C-MYC nanoprobe (n=16), pleural effusion samples incubated with SOX-2 nanoprobe (n=7) and blood samples incubated with C-MYC nanoprobe (n=19). For each sample type, individual data points are shown on the left (red crosses) and box plots are shown on the right. Within each box plot, the blue box represents the events within 25% to 75% of the sample quartile, with the centre line representing the sample median and the middle box representing the mean. Red crosses to the left of the box plots represent the individual data points, with red dots within the box plot representing outlier data points.

This analysis shows that pleural effusion samples display a wider range of fluorescence intensities, with C-MYC incubated pleural effusions displaying a range from 0.06% to 3.21%, with a median of 0.85%. SOX2 incubated pleural effusions also displayed a wide range of fluorescence intensities detected, with a range from 0.1% to 8.27% and a median of 2.63%. The maximum signal from SOX2 pleural effusion sample is an outlier, as this is much higher than the rest of the SOX2 pleural effusion samples. However, this may be due to a low sample number and performing further analysis with a larger sample pool would be beneficial to identify the potential of SOX-2 as a cancer biomarker for liquid biopsies.

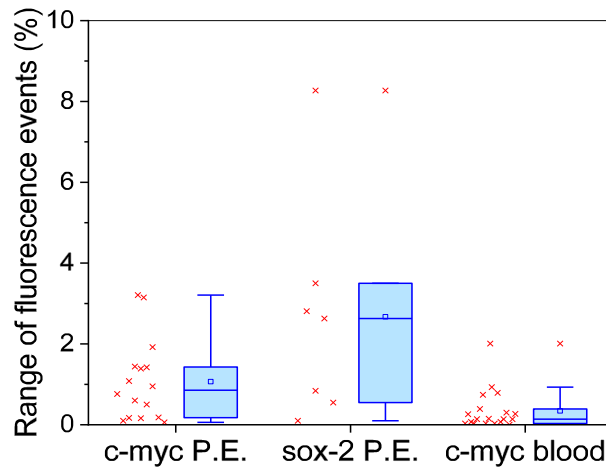


Figure 60. Evaluation of the range of fluorescence events detected after C-MYC and SOX-2 nanoprobe incubation in liquid biopsies.

Pleural effusion (P.E.) samples incubated with C-MYC nanoprobe (n=16) and SOX2 nanoprobe (n=7) and blood samples incubated with C-MYC nanoprobe (n=19) were analysed by flow cytometry and the percentage of fluorescence events detected following nanoprobe incubation are displayed in a box plot. The blue box represents the events within 25% to 75% of the sample quartile, with the centre line representing the sample median and the middle box representing the mean. Red crosses to the left of the box plots represent the individual data points, with red dots within the box plot representing outlier data points. This analysis shows the range of fluorescence events detected with C-MYC and SOX2 targeted nanoprobe. Pleural effusion samples show a wider range of detection as these samples are enriched with cancer cells. Blood samples incubated with nanoprobe displayed a lower percentage of fluorescence events however this demonstrates that cancer mRNAs are still detectable.

Blood samples incubated with C-MYC displayed a much smaller range and lower percentage of fluorescent events, as compared to pleural effusion samples. This is not surprising, as CTC in blood are much rarer than cancer cells in pleural effusions.

The range of fluorescence events in blood samples was from 0.002% to 2.01%, with a median of 0.14%. This method of using nanoprobe and flow cytometry is different from current methods of CTC detection. Current methods have issues with low capture rate, due to size filtration or using antibodies that only capture a subpopulation of CTCs that express the marker of interest. Further studies are required to confirm whether all events detected by nanoprobe flow cytometry are CTCs but these there results are promising, with 18 out of 19 blood sample incubated with the nanoprobe displaying a positive fluorescence population that was above the detection limit.

Some liquid biopsies from cancer patients displayed a low level of fluorescence events. This could be due to the particular cancer not exhibiting high levels of C-MYC mRNA. Although the over expression of C-MYC is a common cancer genetic contributor, it is not over expressed in all tumours. Therefore, multiplexed analysis with multiple different cancer mRNA biomarker targets would strengthen a cancer screening test in liquid biopsies. Furthermore, the low florescence events detected in some samples could also be due to the treatment history of the patient. Although these cancer patients were metastatic, some patients could be responding well to treatment, therefore the cancer would be in remission which would reduce the number of CTCs and cell free ctRNAs available in liquid biopsies.

With the majority of clinical samples coming from breast cancer patients, it is important to understand the expression of C-MYC in breast cancer progression. C-MYC has been widely reported to play a role in breast cancer progression and it has been reported to be over expressed in around 50% of breast tumours (220). Furthermore, highly elevated C-MYC expression has been reported in aggressive breast cancer subtypes and tumours that display drug resistance (221). Therefore, the differential number of fluorescence events seen after nanoprobe incubation in different samples could be due to the disease subtype and genetic composition, which influences the expression levels of the mRNA biomarker.

Current challenges in cancer diagnostics are the length of time, the complex analysis methods and lack of a full diagnostic due to tumour heterogeneity and a lack of individual cell analysis. Current CTC detection methods have been described to have low capture rates and are not specific for all CTCs due to the use of one antibody for capture methods, which results in the loss of CTCs that do not express that marker, and size filtration methods results in high background and unspecific capture of cells. Using nanoprobe for CTC detection may offer a rapid, simple and sensitive cancer biomarker mRNA sensing technique.

A limitation of this work is the complexity in the positioning of the sample gate. This analysis was performed several times to find the most appropriate way to gate individual flow cytometry samples. The gating position is highly important for the final analysis, as a slight change in the gating position could result in a significant change in the percentage of fluorescent events that are captured in the gate.

Firstly, all control and nanoprobe treated samples were gated at intensity 0, with the aim to only collect positive data points in the analysis gate B. However, problems were encountered with this as many samples displayed background fluorescence in the control sample, which resulted in the control gate having a high background count. The second method of analysis was to gate each sample individually on the control sample, at the point where the control gate (gate A) contained no data points. This method also resulted in complications, as some control samples contained fluorescent background outliers, with one or two data points that displayed a high fluorescence intensity. Using this method of gating resulted in a large loss of data from the nanoprobe treated sample.

The method of analysis that was found to be most appropriate for the dot plot analysis was gating each sample around the main bulk of the control sample population. This method allowed tailored analysis for each sample and allowed the inclusion of a low background signal of control outlier data points. This allowed the fullest analysis of data points that were contributed by the nanoprobe, while taking into account any background signal.

For clinical use, a defined fluorescence intensity threshold value would be required in order to determine if a sample is nanoprobe positive or nanoprobe negative. In order to define this value, and the most appropriate method of analysis, a clinical trial would be required. This would include a large population of healthy patients, to determine the background signal of the nanoprobe, and a large population of cancer patients of different disease types and disease stages to determine the nanoprobe fluorescence intensity in different sample types.

Another limitation of this work is the unknown uncertainty behind each measurement. A valuable parameter for these clinical sample measurements would be the coefficient of variation (CV) value, which measured the uncertainty of a flow cytometry measurement. The CV value is dimensionless and does not depend on the intensity of the measured population, therefore it can be used to compare the variation between different sample populations. The CV value represents the percentage of data spread, from the mean value of the population. This would provide valuable information when comparing values of low intensity, and when comparing different samples with similar values, to determine if the uncertainty values make the difference significant, or whether the sample values are comparable.

This is the first investigation on the potential of nanoprobes to detect cancer biomarkers in human blood, and measurement and analysis techniques gained from this experience is hoped to shape and improve future nanoprobe clinical investigations.

7.4. Conclusions

This investigation has shown the ability of nanoprobes to detect cancer mRNA biomarkers, C-MYC and SOX2, within cancer patient liquid biopsies. These results demonstrate that in each cancer liquid biopsy sample, the nanoprobe treated samples exhibited a fluorescence population that was not present in the control samples. In metastatic pleural effusion samples treated with C-MYC nanoprobes, the fluorescence population ranged from 0.06% to 3.21%, with an average of 1.06%

of the total sample population. In blood samples from metastatic cancer patients, the fluorescence population ranged from 0.002% to 2.01%, with an average of 0.34%.

Although pleural effusion samples display a higher fluorescence, due to the higher proportion of cancer cells, pleural effusion samples do not present an ideal cancer diagnostic technique for early cancer detection, as they are typically a symptom of late stage cancer. However, metastatic pleural effusions are a rich source of cancer cells and these samples provided a valuable test for the ability of nanoprobe to detect cancer mRNA biomarkers within patient liquid biopsies.

The results of utilising blood samples as liquid biopsies for CTC detection are very promising. All blood samples from cancer patients displayed a fluorescence population that was not present in the control sample. The results show that cells expressing mRNA biomarkers can be detected down to 0.002%, which is a detection of 2000 events per million. This confirms the capability of using nanoprobe to detect target mRNA in liquid biopsies in cancer patients by flow cytometry.

One pleural effusion sample was obtained from a non-cancer patient. Following nanoprobe incubation in this sample, no fluorescence signal was observed, and the sample was identical to the untreated control sample. Although only one non-cancer sample was tested, this provides a positive preliminary result that the nanoprobe did not exhibit non-specific opening when no cancer biomarker was present. Unfortunately, no blood samples could be obtained from non-cancer patients. This would have been a good control to observe if the nanoprobe displayed a non-specific opening fluorescence in a non-cancer patient or if the nanoprobe remained closed.

The results reported in this chapter are significant as applying nanoprobe to liquid biopsies provides a sensitive method to rapidly detect cancer mRNA biomarkers on the individual cell level. This would provide the potential for cancer screening, diagnostics, treatment guidance and follow-up to monitor the disease progression.

8. Investigating the ability of nanoprobe to detect target mRNA in exosomes

8.1. Introduction

Exosomes are extracellular nanovesicles that range from 30 – 150 nm in diameter and secreted from nearly all mammalian cell types. Exosomes are formed by the fusion of late endosomes, which carry cytosolic cellular components, with the plasma membrane and are released into the extracellular environment (222). Both healthy cells and tumour cells have been reported to secrete exosomes, and it is thought they play an important role in cellular communication through the transfer of molecules such as proteins, RNA and DNA between cells (223). Exosomes have been found in a range of biological fluids, such as plasma, urine, saliva, cerebrospinal fluid, amniotic fluid and pleural effusions (224).

Tumour exosomes have been shown to be crucial mediators of tumour cell communication and have been shown to promote tumour growth and metastasis. Tumour exosomes have been shown to transfer oncogenic materials that promote tumorigenesis in recipient cells. This includes the spreading of mutations that promotes cell growth, angiogenesis and cell invasion (225).

As well as promoting tumour progression, tumour exosomes are carriers of genetic biomarkers. Cell free ctDNA and ctRNA has been recognised as a potential method for non-invasive cancer biomarker detection. Circulating tumour DNA and RNA has been shown to exist in the bloodstream and a recent study demonstrated that 93% of ctDNA that exists in the plasma is located within exosomes (226). Genetic material that are transported in exosomes are protected from enzymatic degradation during circulation, which makes them stable biomarkers in the blood. Furthermore, exosome shedding has been found to be enhanced in the serum of cancer patients, as compared to healthy individuals (227). Therefore, the detection of tumour exosomes provides an opportunity for sensing genetic cancer biomarkers in liquid biopsies (228).

Studies isolating tumour exosomes from serum have found that they exist at a concentration of 10^{12} per ml of blood. The contents of serum exosomes were investigated, and the RNA content was quantified by sequencing. This found that RNA content is high in exosomes, with mRNAs making up ~10% of the total cargo (229). It has also been estimated that exosomes have the capacity to carry around 10,000 nucleotides per exosome (230). Other studies investigating exosome capture have reported that serum exosome concentration ranges between 10^7 and 10^9 per ml of blood (231). Therefore, biological fluids such as blood contain exosomes that carry a rich source of information that may provide clinical diagnostic biomarkers.

A study investigating the detection of exosomes in early stage and late stage cancers have found ctDNA was detectable in multiple different cancer types and at different disease stages. Using PCR, ctDNA was detectable in over 75% of blood samples from patients with metastatic ovarian, colorectal, breast and pancreatic cancers (232). Furthermore, ctDNA was detected in 73%, 57%, 48% and 50% of patients with localised colorectal, gastroesophageal, pancreatic and breast cancers, respectively. It was also reported that in early stage cancers, ctDNA was often present without detectable CTCs, suggesting that the two types of circulating tumour biomarkers are produced through different mechanisms and that ctDNA may provide a biomarker for early stage cancers.

In this investigation, the potential of nanoprobe to detect target cancer biomarker mRNA in exosomes extracted from cell lines was demonstrated, providing the potential for nanoprobe to detect cell free circulating mRNAs within liquid biopsies. Exosomes were extracted from Hek and PC3 cell lines and the size and concentration of exosomes were measured by the gold standard, NanoSight (233). The potential of the nanoprobe to detect C-MYC mRNA within exosomes derived from cancer cell lines was demonstrated through steady state and time resolved fluorescence spectroscopy.

8.2. Sample preparation

8.2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) (D5796), non-essential amino acid solution (M7145), sodium pyruvate (S8636), penicillin-streptomycin (P4333) were purchased from Sigma- Aldrich. Total Exosome Isolation (from cell culture media) (4478359) was purchased from Fisher Scientific.

8.2.2. Exosome extraction

Exosomes were extracted from cell lines using the Invitrogen Total Exosome Isolation (from cell culture media) solution. 3 million cells were seeded into T-25 cell culture flasks and were grown in FBS free media for 24-hours. The cell media was then collected and centrifuged for 2000 x g for 30 minutes at room temperature to remove cell debris. The supernatant was then transferred into a fresh tube and 0.5 volumes of total exosome isolation reagent was added. This solution was mixed well before being incubated at 4°C overnight. The following morning, the sample was centrifuged at 10,000 x g for 1 hour at 4°C. The supernatant was discarded and the pellet containing exosomes was resuspended in 1 ml PBS and stored at 4°C.

8.2.3. Measuring exosome concentration by NanoSight

Once exosomes were isolated, the size and concentration of each sample was measured on a NanoSight LM10 within 24-hours. This uses nanoparticle tracking analysis (NTA) software version 2.2 to count, size and visualise nanoparticles in a liquid suspension. The NanoSight comprises of a microscope, a CMOS camera and a sample viewing unit. A laser is used to illuminate particles that are suspended in solution in the viewing platform and the sample is advanced through the viewing platform by a syringe through the flow chamber. The NTA software identifies and then tracks the movement of individual particles on a frame-by-frame basis, using Brownian motion. The velocity of particle movement is used to calculate particle size by applying the Stokes-Einstein equation. A total of 3 measurements were performed for each sample, with a measurement time of 60 seconds each. After

each measurement the sample was advanced to sample a different area. NTA post acquisition settings were optimized and remained constant between sample. Each video was analysed to give the mean, mode, and median particle size, along with an estimate of the concentration.

8.2.4. Steady state fluorescence spectroscopy

The fluorescence emission spectrum was measured using a Fluorolog-3 spectrofluorometer with excitation at 638 nm and a 5 nm slit. The measurement range was set from 650 nm to 750 nm to collect the emission from cy5. Each sample measurement was repeated 5 times and averaged to obtain an emission intensity graph. Measurements were taken with nanoprobe alone and then 2 hours after incubation with exosomes at a ratio of 10 nanoprobe: 1 exosome.

8.2.5. Time resolved fluorescence spectroscopy

The fluorescent lifetime of the probe was measured by time correlated single photon counting on an IBH Fluorocube fluorescence lifetime system (Horiba Jobin Yvon IBH Ltd., Glasgow, UK). A pulsed light emitting diode of 638 nm was used as the excitation source at 1 MHz. MHA-GNRs at OD= 0.2 were measured as a prompt at 638 nm emission and 0°. The nanoprobe was measured at emission 665 nm and at 55° to minimise polarisation. Nanoprobe was incubated with exosomes at a ratio of 10:1, and incubated for 2 hours at 37°C, before time resolved fluorescence spectroscopy was repeated.

8.3. Results

8.3.1. Investigating the ability of nanoprobe to detect exosomes isolated from cell lines by fluorescence spectroscopy

To examine the ability of nanoprobe to detect mRNA biomarkers within exosomes, exosomes were extracted from PC3 cells, a cancer cell line which overexpress the mRNA of the cancer biomarker C-MYC, and Hek cells, a non-cancer cell line which has low expression of C-MYC. Exosomes were extracted from 3 million cells from

each cell line, using the Total Exosome Isolation reagent. This reagent provides a simple and rapid procedure in which to concentrate and extract intact exosomes from cell culture media. This reagent functions by forcing less-soluble components of the media, exosomes, out of solution, which allows them to be collected after centrifugation.

Following exosome isolation, the concentration of Hek and PC3 exosomes were measured, in order to use these samples in downstream analysis for direct comparison. The concentration of GNRs in a C-MYC nanoprobe sample were also measured, to be able to incubate the nanoprobe at a known ratio with exosomes. The concentration of each sample was measured on a NanoSight LM10 which uses nanoparticle tracking analysis to detect the number, size and concentration of particles within a solution.

The NanoSight manual defines the optimal number of particles in each frame to range from 20-60. This is because statistically, less than 10 particles do not provide enough data points to track, whereas over 100 particles saturate the image. Both exosomes samples were diluted 1:500 in PBS and the nanoprobe sample was diluted 1:400,000. The average number of particles that were recorded in the PC3 exosomes image was 53, while Hek exosomes displayed an average of 45 particles and the nanoprobe sample displayed an average of 17 particles. This shows that the particle concentration in each measurement was ideal for an accurate concentration analysis.

The size distribution histogram for each sample is shown in Figure 61. The main peak for Hek exosomes was shown at 195 nm (Figure 61a), while PC3 exosomes displayed a peak size of 175 nm (Figure 61b). This is consistent with published results, which reported exosomes sizes from cell lines ranging from 120 – 180 nm (234). A larger size peak can be observed in both samples at ~380 nm and ~550 nm, which could indicate exosome clumping or the presence of microvesicles, which are a larger subset of extracellular vesicles. For the nanoprobe sample, a size peak was observed at 85 nm (Figure 61c). The NTA software is optimised for spherical

particles and due to the high scattering of GNRs it is challenging to obtain accurate GNR size measurements, therefore the GNRs appear larger than the actual size. However, the concentration calculation is still valid, which is what was used to calculate how much nanoprobe to add to the exosome samples.

The concentration calculated for each exosome sample and the nanoprobe sample by the NTA software and the absolute concentration for each sample after the dilution factor was calculated (

Table 13). This shows that amount of Hek and PC3 exosomes produced by cell lines were of a similar magnitude, with Hek cells producing 4.39×10^{11} and PC3 cells producing 5.2×10^{11} exosomes per ml. The GNR concentration in the nanoprobe sample was very high, with 6.56×10^{13} GNRs per ml.

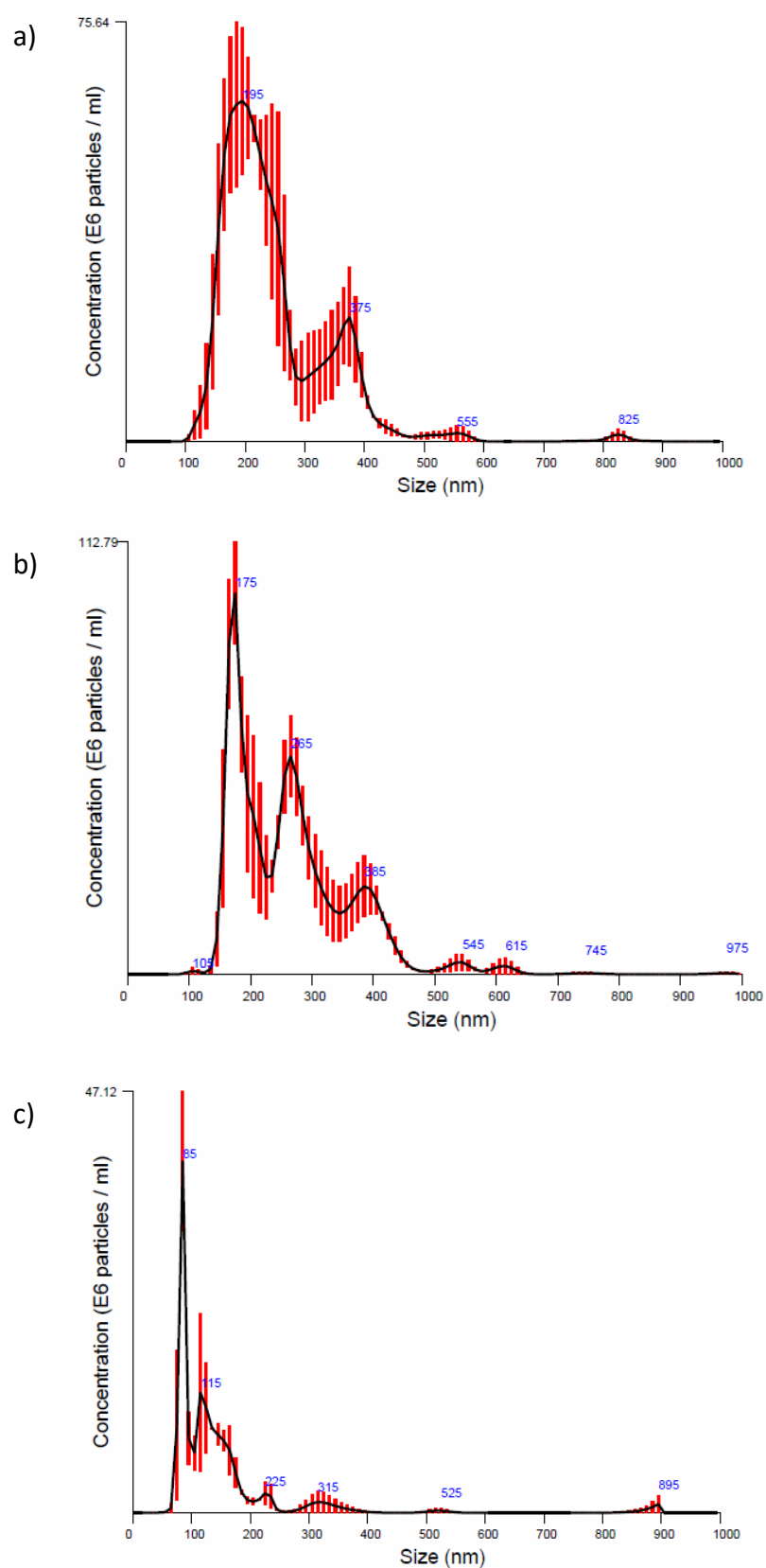


Figure 61. Size distribution of exosomes and gold nanorods by NanoSight.

NanoSight size measurements of a) Hek exosomes b) PC3 exosomes and c) gold nanorods.

NanoSight samples	Concentration	Dilution	Final concentration
Hek exosomes	8.8×10^8	1:500	4.4×10^{11}
PC3 exosomes	1.04×10^9	1:500	5.2×10^{11}
Nanoprobe	1.64×10^8	1:400,000	6.56×10^{13}

Table 13. The concentration of exosomes and gold nanorods measured by NanoSight.

Once the exosome and GNR concentration were known, the nanoprobe sample was incubated with Hek and PC3 exosomes, to determine the ability of the nanoprobe to enter exosomes and detect C-MYC biomarker mRNA. Both Hek and PC3 exosomes were diluted to a concentration of 4.4×10^{11} in 100 μ l of PBS and the fluorescence emission of exosome samples alone was measured in a quartz microcuvette, with excitation at 638 nm and emission was collected between 650 – 750 nm. The emission of the nanoprobe was also measured alone with the same measurement conditions. The C-MYC nanoprobe was then incubated with Hek and PC3 exosomes at a ratio of 10 nanoprobe: 1 exosome and the samples were left to incubate for 2 hours at 37°C. The fluorescence emission spectra of Hek and PC3 exosomes alone, nanoprobe alone and exosomes after nanoprobe incubation are shown in Figure 62.

The fluorescence emission of exosomes alone revealed that they are not fluorescent, as they do not present any fluorescence peaks. The fluorescence emission of the C-MYC nanoprobe alone display a low fluorescence background, as the hpDNA is in a closed state. The fluorescence emission of the nanoprobe after incubation with Hek exosomes shows a fluorescence intensity with a small increase in fluorescence, with a 1.3-fold increase, as compared to the fluorescence of the nanoprobe alone. This demonstrates that the majority of the nanoprobe hpDNA has not opened after interacting with exosomes. In contrast, the fluorescence emission of the nanoprobe after incubation with PC3 exosomes displays a significant increase in fluorescence, with a 9.9-fold increase as compared to the nanoprobe alone. This

is an important result, as it is the first time mRNA expression in exosomes has been investigated using fluorescent nanoprobe and it demonstrates that the nanoprobe is capable of specifically detecting mRNA biomarkers within exosomes. The low fluorescence of the nanoprobe after Hek exosome incubation demonstrates the stability of the nanoprobe and further confirms the nanoprobe does not open randomly in the absence of target mRNA.

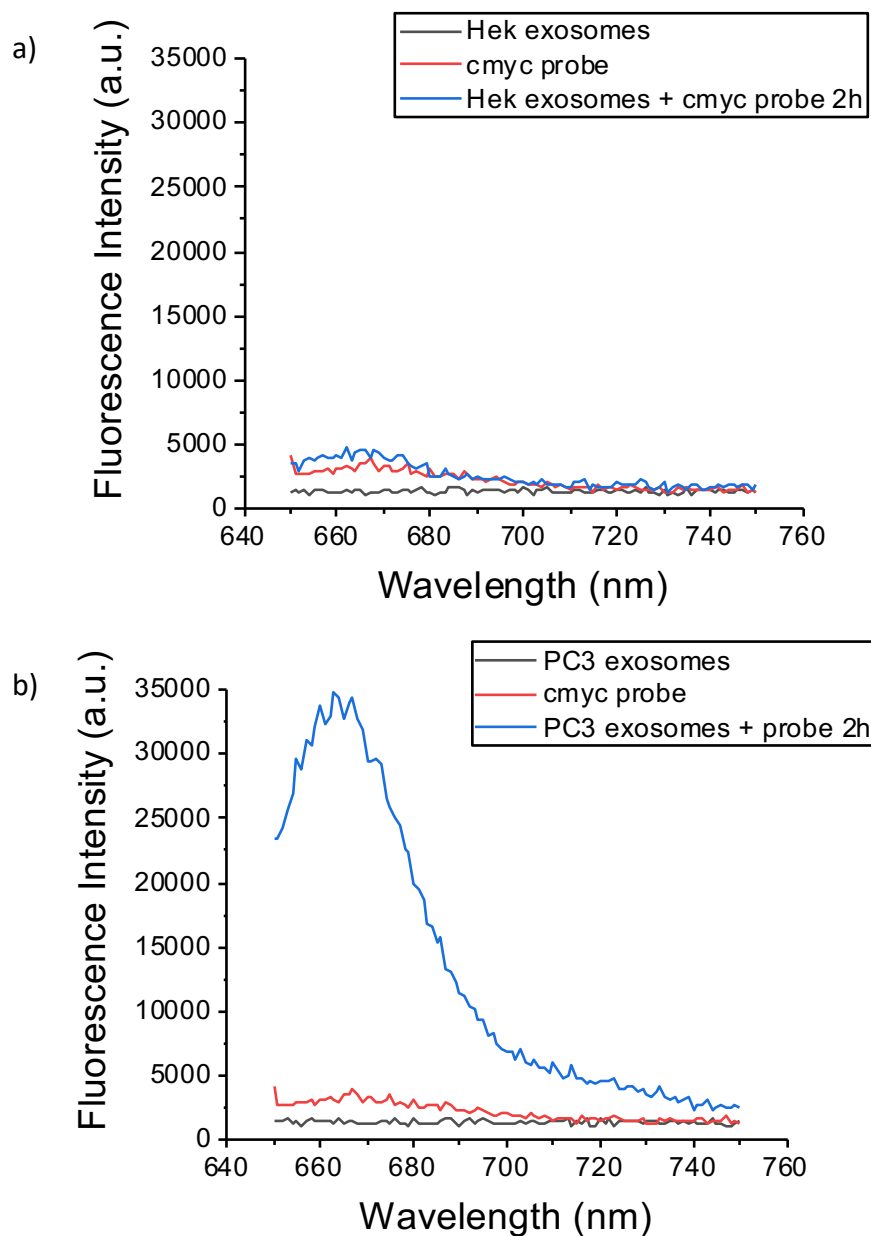


Figure 62. Fluorescence emission spectroscopy of exosomes extracted from Hek and PC3 cells before and after nanoprobe incubation.

The fluorescence emission of a) Hek exosomes alone (black), c-myc nanoprobe alone (red) and Hek exosomes after a 2-hour incubation with c-myc nanoprobe (blue) and b) PC3 exosomes alone (black), c-myc nanoprobe alone (red) and PC3 exosomes after a 2-hour incubation with c-myc nanoprobe (blue). This demonstrates that the nanoprobe is capable of sensing mRNA within exosomes and only fluoresces after incubation with exosomes from target expressing cells.

Time resolved fluorescence spectroscopy was performed on the same Hek and PC3 exosomes samples, following incubation with C-MYC nanoprobe. The fluorescence lifetime was measured with a 638 nm LED excitation source, with emission collected at 665 nm. Figure 63a displays the fluorescence lifetime decay profiles for the C-MYC nanoprobe alone, the nanoprobe incubated with Hek exosomes and the nanoprobe incubated with PC3 exosomes. The fluorescence lifetime decay of free cy5 was also measured. From visualising the decay curves, it can be observed that the nanoprobe alone has the shortest lifetime decay, with a rapid decrease in the histogram. The nanoprobe incubated with Hek exosomes displays a slight increase the decay time but the nanoprobe incubated with PC3 exosomes displays a much longer decay time, which is very close to the decay of free cy5 alone.

The fluorescence lifetime decay curves that contained nanoprobe were fitted with a 3-exponential decay, to extract the 3 different fluorescent components within each sample. These decay components are τ_1 , the quenched cy5 which exists in the closed hpDNA nanoprobe configuration, τ_2 , the scattering from GNRs and τ_3 , the unquenched cy5 which exists in the open hpDNA nanoprobe configuration. The fluorescence lifetime decay values, and the standard deviation in brackets, for each sample are displayed in Table 14. All χ^2 values were close to 1, which represents a goodness of fit.

The nanoprobe alone displays 3 fluorescence lifetime components, τ_1 being the lifetime of quenched cy5 which displays a fluorescence lifetime of 0.55 ns and account for around 3% of the sample. τ_2 was a very short lifetime competent of the scattering of GNRs, which accounts for 91% of the sample. τ_3 represents the fluorescent lifetime of unquenched cy5, which accounts for around 6% of the sample.

Following incubation with Hek exosomes, the fluorescence lifetimes in the sample saw a shift. τ_1 of 0.92 ns which represents quenched cy5 saw a slight increase in the B value to 5%, τ_2 of 72.1 ps, which represents the contribution from the scattering from GNRs decreased to 63.8% and τ_3 of 1.3 ns, which represents the contribution

from unquenched cy5 increased to 31%. This demonstrates that there was some fluorescence from nanoprobe after Hek exosome incubation. Hek cells display low expression of C-MYC mRNA, therefore some nanoprobe hpDNA opening is expected.

However, after the nanoprobe was incubated with PC3 exosomes there was a significant increase in the fluorescence lifetime of the sample. The τ_1 component of quenched cy5 of 0.88 ns saw an increase to 44% contribution, while the contribution of τ_2 , GNRs, decreased dramatically to 4%. τ_3 of 1.58 ns which represents unquenched cy5 within the nanoprobe increased to 52%. This demonstrates that after incubation with PC3 exosomes, the majority of the sample contribution is coming from unquenched cy5, where the nanoprobe is in the open hpDNA conformation.

The fluorescence lifetime of free cy5 functionalised to hpDNA was also measured and analysed with a 2-exponential decay function. This analysis shows that even the free cy5 sample displays two lifetime components, τ_1 with a lifetime of 0.8 ns, which has a contribution of 23.98%, and τ_2 demonstrated a longer component of 1.7 ns, which made up the majority of the sample with a contribution of 76.02%. This result is similar to previously published results of the lifetime components of cy5 functionalised to DNA, which found that the sample had two fluorescence lifetime components. The first component was 0.3 ns and had a 30% contribution, with a second fluorescence lifetime of 1.8 ns that had a 70% contribution (235). The presence of two lifetimes within the cy5-hpDNA sample could be due to self-quenching of the fluorophore when in close proximity to one another.

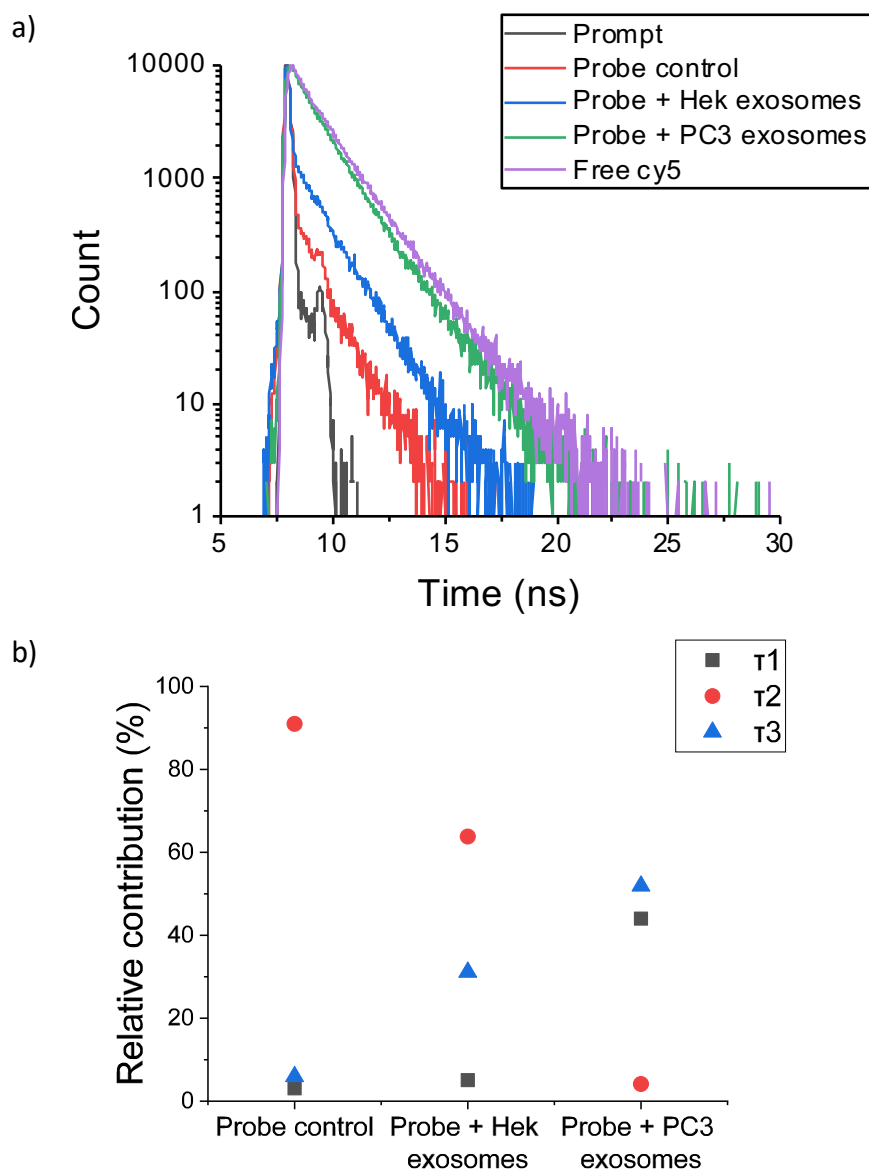


Figure 63. Time resolved fluorescence spectroscopy of C-MYC nanoprobes after incubation with exosomes extracted from Hek and PC3 cells.

a) The fluorescence lifetime decay histograms of C-MYC nanoprobes alone (red), following incubation with Hek exosomes (blue), PC3 exosomes (green) and free hpDNA-cy5 (purple). b) The percentage of relative contribution of each fluorescence lifetime component within a nanoprobe control sample, Hek exosomes incubated with nanoprobe and PC3 exosomes incubated with nanoprobe. The contribution of quenched cy5 (τ_1), the scattering of gold nanorods (τ_2), and the contribution of unquenched cy5 (τ_3) have been plotted. These results show that the contribution of GNRs decreases and the contribution of cy5 increases slightly within Hek exosomes that express low levels of target mRNA and this change is significant in PC3 exosomes that overexpress target mRNA. This demonstrates that the nanoprobe is capable of detecting mRNA biomarkers in exosomes.

	τ_1	B1	τ_2	B2	τ_3	B3	X2
Nanoprobe alone	0.55 ns (0.13ns)	3.09	23.1 ps (2.8ps)	90.98	1.30 ns (59.65 ps)	5.94	1.07
Probe + Hek exosomes	0.92 ns (0.30ns)	5.07	72.1 ps (2.46ps)	63.81	1.30 ns (26.67ps)	31.12	1.15
Probe + PC3 exosomes	0.88 ns (46.64ps)	44	152 ps (19.62ps)	4.13	1.58 ns (10.24ps)	51.88	1.11
Cy5 alone	0.82 ns (29.54ps)	23.98	1.7 ns (58.08ps)	76.02	-	-	1.00

Table 14. Fluorescence lifetime decay fitting analysis for C-MYC nanoprobe before and after incubation with exosomes from Hek and PC3 cells.

The B value relative percentage contribution for each lifetime component of the nanoprobe control sample and the nanoprobe incubated with Hek and PC3 exosomes, are plotted in Figure 63b. This demonstrates that in nanoprobe incubated with PC3 exosomes, the majority of the contribution coming from unquenched cy5, suggesting that the nanoprobe is in an open hpDNA conformation and has bound to target mRNA. The high contribution from unquenched cy5 is not observed after nanoprobe incubation with Hek exosomes, suggesting that some hpDNA is open but the majority of the sample contribution is contributed from the scattering of GNRs.

8.3.2. Investigating the ability of the nanoprobe to detect exosomes at clinically relevant concentrations

Once the ability of the nanoprobe to detect PC3 exosomes had been confirmed, the ability of the nanoprobe to detect clinically relevant concentrations of cancer exosomes was investigated. The concentration of exosomes found in the blood varies in the literature from 10^7 to 10^{12} per ml of blood. In order to investigate the

nanoprobes ability to detect exosomes at physiological relevant concentrations and below, PC3 exosomes were diluted between 10^2 and 10^{10} exosomes per ml and were incubated with 0.1 nM of C-MYC nanoprobe. Each sample was incubated for 2 hours at 37°C, before fluorescence emission was performed. Each measurement was repeated 3 times and the average peak fluorescence emission was plotted for each dilution. The peak fluorescence emission was taken at 665 nm, as this is the peak emission wavelength for cy5.

The peak fluorescence emission for each exosome dilution is shown in Figure 64. The fluorescence emission of 0.1nM of nanoprobe alone in 1 ml PBS was taken as a control and demonstrates the fluorescence background of the nanoprobe. The fluorescence emission was greatly increased when the nanoprobe was incubated with 10^2 exosomes and continued to increase at each increasing concentration of exosomes, with the highest fluorescence emission observed with 10^{10} exosomes. A linear fit was performed between exosome concentrations 10^2 to 10^{10} and this showed a good fit, with the R^2 value of 0.895. This demonstrates that the fluorescence intensity of the nanoprobe increased as the concentration of exosomes increased, which suggests that the nanoprobe fluorescence is proportional to the concentration of target mRNA.

Steady state fluorescence spectroscopy has demonstrated the ability of the nanoprobe to detect cancer mRNA expressing exosomes, at concentrations much lower than clinically relevant concentrations. Furthermore, the fluorescence emission intensity of the nanoprobe increases with an increased concentration of target mRNA, demonstrating the ability of the nanoprobe to differentiate differential expression levels.

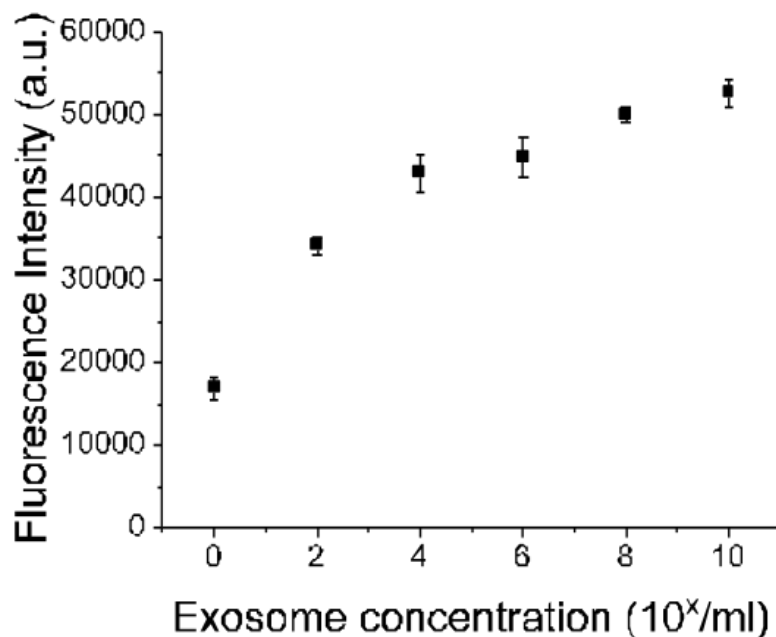


Figure 64. Peak fluorescence emission spectroscopy of the nanoprobe incubated with a PC3 exosome dilution.

The peak fluorescence emission measured for the c-myc nanoprobe alone (0) and after incubation with exosomes ranging from 10^2 to 10^{10} exosomes per ml. The fluorescence intensity of the nanoprobe increased with increasing nanoprobe concentration. This demonstrates that the nanoprobe can detect clinically relevant exosome concentrations and the fluorescence emission intensity is proportional to the target expression.

8.4. Conclusions

Fluorescence spectroscopy measurements have demonstrated that C-MYC nanoprobes are capable of discriminating exosomes derived from C-MYC overexpressing cells as compared to exosomes derived from cells that have low expression of C-MYC, with both fluorescence emission and fluorescence lifetime of

cy5 increasing following incubation with exosomes derived from target mRNA overexpressing cells.

The investigation into exosomes derived from cell lines has demonstrated that nanoprobe exhibit fluorescence only when incubated with exosomes from target expressing cancer cells, and not with exosomes derived from non-cancer cells. This suggests that exosomes can carry mRNA molecules from their cell of origin and that nanoprobe are capable of entering exosomes and detecting the mRNA within.

This investigation also demonstrated that nanoprobe are capable of detecting exosomes at concentrations that are well below clinically relevant concentrations and the fluorescence intensity from the nanoprobe corresponds to the concentration of exosomes within the sample. Furthermore, fluorescence lifetime values can be used to determine the hybridisation state of the hpDNA, based on the extent of fluorescence quenching of cy5.

There is significant evidence in the literature to suggest exosomes carry a high concentration of mRNA from the cell of origin; therefore, nanoprobe could be applied to liquid biopsies to detect target mRNA both within CTCs and within circulating exosomes. Therefore, it is possible that the fluorescence events detected from liquid biopsies are from a combination of CTCs and cell free mRNA within cancer exosomes, however more studies are needed to confirm this.

This is the first time that exosomes from cell lines have been investigated with fluorescent nanoprobe to detect mRNA expression within exosomes. These results are significant as they show that nanoprobe specifically fluoresce in exosomes derived from C-MYC overexpressing cancer cell lines, demonstrating the ability for nanoprobe to detect cancer mRNA biomarkers that are transported within exosomes. For cancer liquid biopsy applications, this provides the potential for nanoprobe to detect cell free circulating cancer biomarker mRNAs within exosomes in the blood.

9. Summary and Future work

9.1. Summary

Early detection of cancer results in improved survival, as the tumour is small, localised and has not developed heterogeneity that induces drug resistance. Cancers that are diagnosed early have a higher chance for successful treatment, which greatly improves patient survival. Current cancer diagnostic from tumour biopsies are invasive, complex and lengthy. Many cancers have an altered mRNA expression, which is a genetic biomarker that can be used to distinguish cancer from non-cancer cells. The aim of this thesis was to develop nanoprobe for cancer biomarker mRNA detection in liquid biopsies.

The work presented in this thesis has reported the synthesis, characterisation and applications of fluorescent GNR nanoprobe for the detection of cancer biomarker mRNAs. This nanoprobe design functions through a GNR functionalised with fluorophore labelled hpDNA that contains a targeting loop against an mRNA biomarker of interest. In the closed nanoprobe configuration, the hpDNA is intact and positions the fluorophore in close proximity to the GNR surface, which facilitates FRET between the fluorophore and GNR and results in fluorescence quenching. Upon hpDNA target mRNA binding, the hpDNA is dissociated, which removed fluorescence quenching and recovers fluorescence. In this design, fluorescence should only occur upon nanoprobe binding to the target mRNA.

In chapter 3, the synthesis of stable and biocompatible GNRs was demonstrated and the functionalisation of GNRs with fluorophore labelled hpDNA molecules has been optimised, using a pH-controlled salt-aging process. The pH dependence of DNA functionalisation was a significant discovery, with nanoprobe produced at pH5 demonstrating an enhanced ability to detect target DNA. The ability to produce nanoprobe that have sufficient and stably bound hpDNA is essential for the nanoprobe function. The low background fluorescence in the closed hpDNA nanoprobe configuration was demonstrated through steady state and time-

resolved fluorescence spectroscopy. The specificity of hpDNA opening dynamics has been demonstrated following complementary DNA incubation, which resulted in a removal of fluorescence quenching and the restoration of cy5 fluorescence.

In chapter 4, the applications of flow cytometry as a measurement technique to detect the fluorescence of nanoprobe in individual cells has been validated. These results demonstrated the ability of flow cytometry side scatter to detect the uptake of GNRs within cells, through GNR scattering, which allowed the optimisation of GNR incubation with cells for maximum GNR uptake. Flow cytometry was also shown to be a sensitive measurement technique for sensing the fluorescence of nanoprobe. Following nanoprobe incubation with mRNA biomarker expressing cancer cells, flow cytometry detected a high fluorescence from these cells, while a low background fluorescence was detected in non-cancer cells. The fluorescence intensity of the nanoprobe in different cell lines by flow cytometry was shown to correlate to mRNA expression levels by qRT-PCR. Furthermore, when target expressing cancer cells were diluted in co-culture with non-cancer cells, a fluorescence population of cancer cells could be clearly discriminated. This demonstrates the ability of nanoprobe to enter cells and come into contact with mRNAs. These results also demonstrated the stability of nanoprobe, as nanoprobe did not exhibit non-specific binding or DNA opening fluorescence in cells that had low expression of target mRNA. This highlighted the potential of nanoprobe to detect cancer mRNAs within CTCs in liquid biopsies with high specificity.

In chapter 5, hpDNA sequences have been designed against a variety of different cancer mRNA biomarkers, all of which have shown preferential binding and fluorescence emission to target DNA sequences. Nanoprobe were also designed against a mutation P53 mRNA biomarker, which contains a one nucleotide difference from wildtype P53 mRNA. The ability of the nanoprobe to discriminate between a one nucleotide mismatch within the hpDNA targeting loop was demonstrated through steady state fluorescence spectroscopy and flow cytometry

studies. These studies also demonstrated that the positioning of the mismatch has an effect on the DNA binding affinity, with a mutation in the centre of the hpDNA having a greatly reduced binding, as compared to the mismatch at the edge of the targeting loop. This highlights the clinical applications of nanoprobe for not only overexpressed mRNA biomarkers, but also for the sensitive detection of mutational mRNA biomarkers.

In chapter 6, fluorescence confocal microscopy of nanoprobe incubated in cell lines have demonstrated nanoprobe uptake and a higher nanoprobe fluorescence in target mRNA target overexpressing cancer cells. FLIM of nanoprobe within cells provides a high contrast imaging agent and the fluorescence lifetime of the nanoprobe can be correlated to the hybridisation state of the nanoprobe hpDNA, which is dependent on target mRNA expression. This investigation demonstrated that the fluorescence lifetime of the nanoprobe is quenched when hpDNA exists in a closed configuration and the fluorescence lifetime can be recovered when the hpDNA is opened. This investigation has shown that fluorescence imaging of nanoprobe provides a rapid and sensitive method for sensing RNA biomarkers at the single cell level.

In chapter 7, the clinical value of nanoprobe in cancer patient liquid biopsies was investigated in a preliminary study. Pleural effusion and blood samples from metastatic cancer patients were analysed by flow cytometry with and without nanoprobe incubation. This demonstrated that nanoprobe treated samples displayed a fluorescence population that was not present in the control samples, with the number of fluorescence events varying between different samples. However, a sample of non-cancer liquid biopsies are required as a control to investigate the background signal of the nanoprobe.

Finally, in chapter 8, the potential for nanoprobe to detect mRNA biomarkers within exosomes was investigated. As well as CTCs, cell free mRNAs provide a valuable source of cancer biomarkers. C-MYC nanoprobe incubated with exosomes derived from Hek cells with low C-MYC expression displayed low fluorescence

intensity and a time resolved fluorescence spectroscopy demonstrated that the majority of cy5 was quenched. Following nanoprobe incubation with exosomes derived from PC3 cells that overexpress C-MYC, a significant increase in both fluorescence emission intensity and fluorescence lifetime was observed. The lack of nanoprobe fluorescence in Hek exosomes supports nanoprobe binding selectivity and demonstrated the low fluorescence background of the nanoprobe.

Therefore, the work in this thesis has supported the use of nanoprobes for the simple, rapid and sensitive technique for cancer mRNA biomarker detection from liquid biopsies.

9.2. Future work

9.2.1. An investigation into the mechanisms of GNR uptake

The results from flow cytometry show an increase in side scatter and an increase in fluorescence specifically in target mRNA expressing cells, combined with the fluorescence imaging results of nanoprobes fluorescing within target mRNA expressing cells, all support the hypothesis that nanoprobes are internalised to cells and come into contact with target mRNA molecules. However, the mechanisms for how GNRs are uptaken and how they escape endosomes to bind to target mRNA is largely unknown.

In order to demonstrate and understand the process of GNR uptake studies on tracking the movement of GNRs from the cell membrane into endosomes and then their movement into the cytosol could be performed. This could be performed through live cell imaging techniques, using fluorescence staining of GNRs and endosomal markers, such as the early endosome antigen 1, to track when GNRs are localised within endosomes and when at which time point and location within the cell they can escape.

Furthermore, endosomes leakage assays have been developed that could be utilised for GNR endosome studies. Calcein is a membrane impermeable fluorescence probe that has been used to study endosomal escape of particles.

Calcein is a self-quenching dye at low pH values, such as conditions inside endosomes. Following particle escape from endosomes, calcein is released into the cytoplasm which removes the self-quenching and results in a fluorescence signal within the cytosol Figure 65 (180). The calcein leakage assay has been reported to be valuable in detecting nanoparticle release from endosomes. This assay, in combination with GNR imaging within cells could correlate GNR location within the cytosol with calcein release. Calcein could be functionalised to GNRs through a linear DNA strand that is at a length that would prevent Calcein being quenched by GNRs.

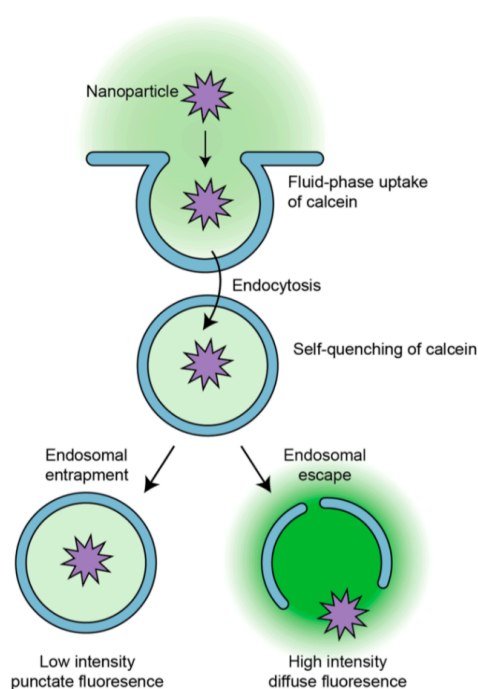


Figure 65. Calcein leakage assay for nanoparticle escape from endosomes.

The release of nanoparticles from endosomes can be detected by the fluorescence of calcein, which is self-quenched inside endosomes and fluoresces when endosome membrane integrity is disrupted. This figure was used with permissions Copyright © 2019, American Chemical Society.

9.2.2. Multiplex flow cytometry measurements:

In this thesis, nanoprobe for multiple different cancer mRNA biomarkers were synthesised. Disease detection using a single biomarker is not feasible as there are no single cancer mRNA biomarker for cancer subtypes due to the disease heterogeneity, therefore the use of a single biomarker within a patient population would result in a screening test with low sensitivity and reliability. However, the combination of multiple mRNA biomarkers could greatly enhance the detection rate of CTCs and cancer cell free mRNAs within liquid biopsies.

In order to perform multiplex flow cytometry analysis, nanoprobe could be designed with GNRs of different longitudinal absorption, with each GNR being functionalised with a hpDNA against a different target mRNA and each hpDNA being functionalised with a fluorophore that emission overlaps with the LSPR of the GNR. Using this method, multiple nanoprobe targeted against different mRNA biomarkers, each labelled with a different fluorophore could all be added to one pleural effusion sample for multi-biomarker expression analysis.

Another way in which multiplexing could be performed is investigating the possibility for multiple different hpDNAs, each targeted against a different mRNA biomarker and labelled with a different fluorophore, to be functionalised to one GNR. In this method, different fluorophores could be used that all overlap with the SPR bands of GNRs, and the sensitivity to detect multiple different mRNA sequences within a sample and the ability to discriminate the different fluorophore emission profiles would be investigated.

Issues in this application would be discriminating the emission profiles of dyes that emission overlap or are spectrally close. Furthermore, if numerous different hpDNAs were to be functionalised to each GNR, the attachment rates of each hpDNA would need to be quantified to ensure each hpDNA is present in the same concentration.

9.2.3. Continuation of clinical pleural effusion samples

Liquid biopsies provide a rich source of cancer mRNA biomarkers and are being investigated for the future of cancer screening and diagnostics. Liquid biopsies provide a non-invasive method for detecting cancer specific biomarkers, such as mRNA with CTCs and exosomes. However, the nanoprobe themselves need to be tested in large controlled clinical studies, with the enrolment of a large population of cancer positive and cancer negative subjects. This would demonstrate the background value of nanoprobe in non-cancer samples, which is important for gating and analysing method for cancer positive samples.

The use of nanoprobe to detect blood-based biomarkers for early cancer screening are still at the early phases of development, and various clinical and preclinical issues need to be addressed. This includes the validation of the mRNA biomarkers of interest in large cancer patient cohorts to confirm the expression levels are detectable from early to late stage of the disease. Having both mRNA biomarkers that are expressed in all cancer disease stages and mRNA biomarkers that expressed at late disease stages would be valuable for multiplexing applications.

Furthermore, the fluorescence population that is detected in clinical samples following nanoprobe incubation need to be confirmed to be signals from CTCs and ctRNAs. CTC validation could be performed through cell sorting onto glass slides and immunocytochemistry staining of the collected cells to determine if they contain cancer markers, which would confirm that they are derived from tumours.

9.3. Conclusion

In this thesis the synthesis and design of novel nanoprobe has been demonstrated and the ability for nanoprobe to specifically detect cancer mRNA biomarkers has been shown. Although the clinical applications of nanoprobe need to be investigated further, these results strongly support the applications of nanoprobe to accurately detect cancer mRNA biomarkers. Nanoprobe provide an exciting and

valuable tool for non-invasive, rapid and sensitive technique for the future of cancer screening in liquid biopsies.

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