Abstract

SmartFlare RNA detection probes are a relatively new commercially available product. They are designed to gain access to the cytosol of live cells after an overnight incubation, without any manipulation or transfection agents. In the cytosol, they detect specific mRNA by an increase in fluorescence that can be monitored in real time with a microscope. The probes consist of a gold core functionalised with specific 18 base-pair oligonucleotides that are hybridised to a short cyanine dye-terminated reporter molecules. The fluorescent dye is quenched due to its close proximity to the gold core. However it is unclear how these SmartFlares actually enter cells and gain access to the cytosol so this was the focus for this study. To investigate this problem we uses both the positive Uptake Control and negative Scramble Control SmartFlares as well as a probe specific to VEGF mRNA. Various live and fixed imaging experiments including co-localisation analysis using HeLa cells gave some unexpected results. Imaging results show a punctate signal for all three SmartFlare probes. This result combined with the co-localisation analysis suggests that the probes are becoming trapped within endosomes rather than reaching the cytosol. Our results show that the SmartFlare RNA detection probes are not working as advertised and further investigation is needed to discover how these probes could gain access to the cytosol.

1. Introduction

Gold nanoparticles that can be synthetically altered by adding different conjugates are seen as hopeful tools with many applications such as drug and gene delivery, cellular imaging/tracking and detection of intercellular molecules such as mRNA (Kim et al., 2012). For such purposes the nanomaterials must combine many different properties including non-toxic, internalised into cells and reach their target, with limited non-specific binding.
1.1 Internalisation mechanisms

Cells internalise surrounding molecules to take up nutrients and probe their environment and this is done by various mechanisms of endocytosis that could be exploited by nanoparticles to enter cells. Clathrin-mediated endocytosis relies on particles binding to specific receptors to allow internalisation (Iversen et al., 2011). The functional unit of clathrin is known as the triskelion and these structures assemble together into regular lattices. In order to associate with the membrane and form vesicles clathrin requires adapter proteins such as AP-2, for cargo can to become internalised (McPherson et al., 2015). Another pathway for internalisation is known as caveolae-mediated endocytosis. Caveolaes are invaginations of the plasma membrane, but their composition, appearance and function varies depending on the cell type. Vesicles are coated with integral membrane proteins known as caveolins and these proteins incorporate with cholesterol rafts also located in the membrane. Together with dynamin and GTP hydrolysis vesicles can become internalised (Pelkmans & Helenius, 2002).

Several other mechanisms of non-endocytic uptake exist. Pinocytosis allows internalisation of nonspecific substances found in surrounding biological fluid. Pockets form in a highly ruffled region of the membrane and are pinched off within the cell to form a vesicle. This pathway could be important for uptake of single nanoparticles with a diameter less than 10 nm (Nuri & Ji-Ho, 2014). Phagocytosis involves active binding and internalisation of materials larger than 0.5 µm such as microorganisms. Only a small subset of mammalian cells can carry out phagocytosis so there is a limited number of cells that could internalise nanoparticles by this pathway (Iversen et al., 2011). Most cells can internalise nanoparticles using one or a few of these pathways, but the size, shape and surface chemistry of the nanoparticles will affect which mechanism is used for internalisation.
1.2 Endosomal escape mechanisms

To be of use in the cell cytoplasm, gold nanoparticles need to be able to escape endosomes in order to reach their target. There are numerous mechanisms described for endosomal escape but they all require an initial destabilization of endosomal membranes, which occurs because of acidification that happens during endosomal maturation. Ionic nanoparticles could interact with the charged phospholipids in the endosomal membrane, this is thought to induce a ‘flip-flop’ bringing the cytosolic-facing phospholipids into the luminal face of the endosome. The stabilisation of the charges causes membrane destabilisation leading to the leakage of the contents (Martens et al., 2014). Another escape mechanism results from some of the internalised molecules having the ability to buffer ions such as $\text{H}^+$, this leads to an entry of water into the endosomes to restore the balance. This causes the compartment to swell and eventually burst due to its destabilised membrane (Martens et al., 2014).

1.3 SmartFlare RNA Detection Probes

SmartFlare RNA detection probes claim to have the desired properties of being able to be taken up into cells without addition agents, are non-toxic to cells and very interestingly they are apparently able to gain access to the cytosol to detect specific mRNA in live cells. It is also claimed that after the probes have detected mRNA they exit the cells via exocytosis leaving the cells unaffected able to carry out further assays (Merk Millipore, 2015). These probes are produced by Merk Millipore and are the commercially available adaptation of the NanoFlare technology developed by the group of Prof Chad Mirkin at the Northwestern University in Illinois. SmartFlares are composed of a 13 nm gold nanoparticle core, which are densely functionalised with specific 18 base-pair thiolated oligonucleotides. The oligonucleotides are then hybridised with short cyanine dye-terminated reporter molecules. The fluorescence of the reporter molecules is quenched by the gold core nanoparticle until the
gold-attached oligonucleotides hybridise with its target mRNA, which displaces the reporter allowing it to fluoresce showing mRNA detection (Prigodich et al., 2009) (Figure 1).

Two controls are also available known as Uptake and Scramble controls. The uptake control is designed as a positive control as the reporter molecule is far enough away from the gold core not to be quenched. Thus, it should constantly fluoresce providing an estimate of overall cell uptake of the SmartFlares. As the scramble controls have no endogenous mRNA with which to hybridise, they are meant to give data on background fluorescence and non-specific release of probe. After we had started this project the company have made available a third control available known as Housekeeping SmartFlares. These are designed to detect an abundant housekeeping gene within your cells and it therefore acts as a positive control (Merk Millipore, 2015).

To investigate the mechanisms of entry of SmartFlares and their potential for intracellular RNA detection, we ordered a Cy5-vascular endothelial growth factor (VEGF) specific SmartFlare as well as the Cy3-Uptake and Cy3-Scramble control SmartFlares. VEGF was selected as the target because it’s transcript level increases in cells as part of a well characterized response to hypoxia. The increase is aided by hypoxia inducible factor-1 (HIF-1). Active HIF-1 is a heterodimer of α and β subunits and levels of HIF-1α are kept low.

**Figure 1:** Gold nanoparticle core functionalised with specific oligonucleotides that are hybridised with short cyanine reporter sequences. Upon entry into the cell and binding of target mRNA there is release of the reporter. The figure has been adapted from Seferos, et al. 2007.
by oxygen-dependent prolyl hydroxylases (PHDs). When a cell becomes hypoxic, PHD levels are reduced, so more HIF-1α is present to dimerise with HIF-1β forming the active heterodimer that acts as a VEGF transcription factor (Bagnall et al., 2014).

Dimethyloxalylglycine (DMOG) is a PHD inhibitor, which mimics the hypoxic state of the cell to increase the levels of HIF-1α that leads to more VEGF in cells. We used DMOG to vary the mRNA levels of VEGF mRNA in the cells (Poynter et al., 2011). For comparison with a traditional method of detecting mRNA we also carried out quantitative polymerase chain reaction (qPCR) using a DMOG treatment.

These SmartFlares have great promise as a new novel way of detecting mRNA within live cells. However there is little information available as to how these SmartFlares enter cells and even less information as to how they gain access into the cytosol to detect the mRNA. Herein, I will report our attempts to use these SmartFlare probes with HeLa cells imaged both live and fixed. Also I will show some of the issues we had, including a lack of endosomal escape and similar signals seen for all three SmartFlares including the negative Cy5-Scramble Control, which is shown by constant punctate signals of similar intensity from the SmartFlares and co-localisation of the SmartFlares with endosomal antibody markers.

2. Open Research

As a part of this project we decided to produce an open science notebook to allow researchers elsewhere to see what experiments we were conducting and the results we obtained in close to real time. The idea of the notebook was to create a collaborative environment were anyone could share their knowledge and interpretations as a continual peer-review process. Also it is hoped that putting this knowledge out on the web could encourage individuals become more
interested and even inspired by the work. A Wordpress blog (accessible at https://raphazlabcommons.wordpress.com) was selected as our way to display data as it is fairly easily accessible to anyone and allows comments enabling interactions. Furthermore, we have also allowed access to our original data using the records management system OMERO. In order to control the unattributed use of the data provided on the blog, we chose to licence the blog and data using a CC-BY Licence that allows anyone to share, adapt and reuse the content as long as acknowledgment is given to the original source (see http://creativecommons.org/licenses/by/4.0/ for more details). At the time of writing this report, the open notebook has been seen 700 times with 300 views from the UK, 140 from the USA and 100 from France.

3. Methods

3.1 Cell culture

HeLa (human cervical cancer) cells were grown in minimum essential medium (MEM) which contained 10% foetal bovine serum (FBS), 1% nonessential amino acids (NAA) and 1% penicillin and streptomycin and were maintained at 37°C in 5% CO₂.

3.2 Quantitative PCR (qPCR) analysis

VEGF mRNA was the gene of interest for the experiment and cyclophilin A was used as the control housekeeping gene. SK-N-AS human neuroblastoma cells (ECACC No. 94092302) were grown in MEM containing 10% FBS and 1% NAA and were maintained at 37°C in 5% CO₂. For the DMOG treatment 0.5 mM of DMOG was added to the culture medium for 24 or 72 hours. For the cultured cells qPCR was carried out as described by Bagnall et al. (2013) at the University of Liverpool.
3.3 Re-suspension of SmartFlares

Each lyophilized SmartFlares sample was re-suspended in a sterile fume cupboard by the addition of 1 mL nuclease free water. These stock solutions were maintained at room temperature protected from light as recommended by the manufacturer.

3.4 Live cell imaging

HeLa cells were seeded at the recommended 500,000 cells per well of a 35 mm glass-bottomed dish 3 hours before addition of experimental mixtures. As recommended 20 µL of each SmartFlare was separately added to the standard medium that also contained 1 mg/mL 10 kDa lysine fixable fluorescein (FITC)-conjugated dextran to give a 1 mL final volume. FITC dextran was used as a positive uptake control. If the cells were undergoing a DMOG treatment 15 µL of stock 100 mM DMOG was also added to the medium. Cells were incubated with the SmartFlares overnight (18 hours) at 37°C in 5% CO₂. Before imaging cells were washed with phosphate buffered saline (PBS) and fresh medium was replaced. Cells were imaged live (at 37°C, 5% CO2) on a Zeiss Multiphoton LSM510 using a 63x (Plan-Apochromat 1.4NA oil) objective. The 488nm, 561nm and 633nm lasers were used to image the FITC-dextran, Cy3-VEGF SmartFlare and Cy5-Control SmartFlares respectively. The scan mode was set to 1024 x 1024 using a 4x averaging line scan. There was also a transmitted-light image captured on the 488nm line.
3.5 Fixed cell imaging

Table 1: Cellular growing conditions and antibody staining used for fixation imaging.

<table>
<thead>
<tr>
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<th>Cy5-Uptake Control</th>
<th>Cy5-Scramble Control</th>
<th>Cy3-VEGF</th>
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<tr>
<td><strong>Anti-LAMP1</strong> (lysosome marker) primary antibody</td>
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<td><strong>Anti-Transferrin receptor (recycle compartment marker) primary antibody</strong></td>
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<td><strong>Anti-Rabbit Alexa555 secondary antibody</strong></td>
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<td><strong>Anti-Rabbit Alexa647 secondary antibody</strong></td>
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Cell samples were prepared and incubated overnight (18 hours) as previously explained. The samples were washed with PBS then fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature and then permeabilised with 0.1% TritonX-100: 1% bovine serum albumin (BSA):PBS for 60 minutes at room temperature. After PBS washes the cells were incubated with either anti-LAMP1 (1:500, ab24170, Abcam) or anti-transferrin receptor (1:500, ab84036, Abcam) for 60 minutes at room temperature covered from light. After which Goat anti-Rabbit IgG Alexa555 (1:1000, A-21428, Life technologies) or Goat anti-Rabbit IgG Alexa647 (1:1000, A-21244, Life technologies) were respectively added to the correct SmartFlare for another 60 minutes at room temperature covered from light and then washed with PBS leaving 1 mL on the cells. Again the cells were imaged on the Zeiss Multiphoton LSM510 using a 63x (Plan-Apochromat 1.4NA oil) objective. The 488nm, 561nm and 633nm lasers were used to image the FITC-dextran, VEGF SmartFlares and control SmartFlares respectively. The scan mode was set to 1024 x 1024 using a 4x averaging line scan. There was also a transmitted-light image captured on the 488nm line.
3.6 Co-localisation Analysis

Co-localisation analysis was carried out on the program Fiji Is Just ImageJ using the plugin Just Another Co-localization Plugin (JACoP). Within the plugin Mander’s coefficient was used on the threshold values to calculate the co-localisation of the SmartFlares with the antibodies in the presence or absence of DMOG treatment (Bolte & Cordelieres, 2006).

4. Results

4.1 Quantitative PCR (qPCR) analysis

As SmartFlares are designed to detect mRNA within cells qPCR was carried out as a comparison to currently used methods. The analysis was carried out on SK-N-AS cells that were treated for 1 or 3 days with 500nM DMOG.

![Figure 2](image_url)

**Figure 2:** Fold increase of VEGF mRNA in SK-N-AS treated for 1 or 3 days with 500 nM dimethyloxalylglycine (DMOG).

The qPCR analysis showed an increases of 13 and 34-fold of *VEGF* mRNA for the 1 or 3 day treatment with DMOG. DMOG treatment can therefore be used as a positive control for our experiments to ensure there is a detectable increase of *VEGF* mRNA within cells for the Cy3-VEGF SmartFlare to detect.

4.2 Live Imaging of Cy5-Uptake Control SmartFlares
Cy5-Uptake control SmartFlare are positive controls where the reporter molecule are at a distance away from the gold core and therefore not to be quenched i.e. Cy5 should constantly fluoresce allowing the visualisation of the SmartFlares taken up into cells. Since these SmartFlares should fluorescent constantly we decided to use this SmartFlare for an initial imaging experiment along with the FITC-dextran as an endocytic pathway marker.

After imaging it appeared that the majority of the HeLa cells had taken up the FITC-dextran showing that the cells are carrying out constitutive endocytosis. The signal appears to be relatively punctate suggesting that the dextran is contained within endosomes (Figure 3A). Unlike FITC-dextran, Cy5-Uptake Control SmartFlares were only taken up in some but not the majority of cell (Figure 3B-C).
When the channel intensities were scaled up to increase lower signals it was revealed that the signal appeared to be largely punctate with some diffuse signal (Figure 6). This mostly punctate signal would again suggest that the SmartFlares are contained within endosomes.

**Figure 4.** HeLa cells loaded with Cy5-Uptake Control SmartFlare for 18 hours with the dynamic range of 1-900 of 4096.

### 4.3 Live Imaging of Cy5-Uptake, Cy5-Scramble Controls and Cy3-VEGF SmartFlares

After establishing the required imaging protocol both the Cy5-Scramble, which is the negative background control, and the Cy3-VEGF SmartFlares were reconstructed. For consistency the protocol used was identical to the previous experiment except for the use of a 561 nm laser line for imaging the Cy3-VEGF SmartFlare.

**Figure 5.** HeLa cells loaded with 10 kDa FITC-dextran and Cy5-Uptake Control SmartFlare for 18 hours with the full dynamic range of intensity (1-4096). **A)** Overlay image of 10 kDa FITC-dextran (green) and Cy5-Uptake Control SmartFlare. **B)** Cy5-Uptake Control SmartFlare only.
The images obtained for the Cy5-Uptake control SmartFlare showed very similar results to the previous experiment with the HeLa cells appearing to constitutively take up the FITC-dextran whereas only a subset of cells take up the Cy5-Uptake Control SmartFlare. Also there is a relatively punctate signal from both the FITC-dextran and the Cy5-Uptake Control SmartFlare suggesting they are likely contained within endosomes (Figure 5).

Figure 6. HeLa cells loaded with 10 kDa FITC-dextran and Cy3-VEGF SmartFlare for 18 hours with the full dynamic range of intensity (1-4096). A) Overlay image of 10 kDa FITC-dextran (green) and Cy3-VEGF SmartFlare. B) Cy3-VEGF SmartFlare only.

Comparable images were obtained from the Cy3-VEGF SmartFlare. The signal from the SmartFlare was not present in all of the cells unlike the FITC-dextran showing the differential uptake of the SmartFlare. Similarly the signal appears fairly punctate proposing its location is within endosomes (Figure 6).
We observed yet again a punctate signal similar to the Cy5-Uptake Control SmartFlare that implies containment within endosomes (Figure 7). The punctate signal was rather unexpected as the SmartFlare is sold as a negative control, which has no endogenous mRNA target, to estimate the background fluorescence for the SmartFlares.

4.4 Fixed Imaging of SmartFlares co-localisation with Antibody Markers

In the previous experiments the SmartFlares punctate signal suggested endosomal localisation, to further investigate if they were co-localised into endosomal compartments we carried out immunofluorescent staining with specific antibodies. The two antibodies selected for the experiment were anti-LAMP1 for labelling lysosome compartments and anti-TFN-R as a marker for recycling compartments. FITC- dextran was used again in this experiment as a marker of the endocytic system.
4.4.1 Localisation with LAMP1

Unfortunately for this imaging there appears to have been some issues with uptake of the Cy3-VEGF SmartFlare samples as there is low uptake of FITC-dextran and this is inconsistent with previous experiments (Figure 8A). However looking at the merge images for the other samples there appears to be some co-localisation taking place (Figure 8B-C).

Figure 8. HeLa cells loaded with SmartFlares for 18 hours and stained with LAMP1 lysosome marker with the full dynamic range of intensity (1-4096). Images are shown as separate grey scales and on a merge image of dextran (green), LAMP1 (blue) and SmartFlare (red). A) Cy3-VEGF SmartFlare. B) Cy5-Scramble Control SmartFlare. C) Cy5-Uptake Control SmartFlare.
4.4.2 Localisation with TFN-R

The same Cy3-VEGF cell samples were used for the TFN-R antibody staining and these appear to have also had lower uptake than usual of the FITC-dextran (Figure 9A). But again there appears to be some co-localisation that requires further investigation (Figure 9).

Figure 9. HeLa cells loaded with SmartFlares for 18 hours and stained with TFN-R lysosome marker with the full dynamic range of intensity (1-4096). Images are shown as separate grey scales and on a merge image of dextran (green), TFN-R (blue) and SmartFlare (red).
A) Cy3-VEGF SmartFlare.
B) Cy5-Scramble Control SmartFlare.
C) Cy5-Uptake Control SmartFlare.
4.4.3 Effect of DMOG

As previously explained there appears to of been poor uptake of the FITC-dextran and Cy3-VEGF SmartFlare with the –DMOG samples (Figure 13). This means that comparison between the two samples is not appropriate, but the signals from the +DMOG sample appear to be punctate which is still consistent with previous results.

![Figure 10. Effect of dimethyloxalylglycine (DMOG) on HeLa cells loaded with Cy3-VEGF SmartFlare for 18 hours with the full dynamic range of intensity (1-4096).](image)

![Figure 11. Effect of dimethyloxalylglycine (DMOG) on HeLa cells loaded with Cy5-Scramble Control SmartFlare for 18 hours with the full dynamic range of intensity (1-4096).](image)

![Figure 12. Effect of dimethyloxalylglycine (DMOG) on HeLa cells loaded with Cy5-Uptake Control SmartFlare for 18 hours with the full dynamic range of intensity (1-4096).](image)
Treatment with DMOG, in a qualitative view, doesn’t appear to affect the intensity or the localisation of the SmartFlares, as the signals appear to have a similar brightness and they are still punctate suggesting they are still contained within endosomes.

4.4.4 Co-localisation Analysis

To quantify the antibody staining images presented in Fig. 8-9, a co-localisation analysis was carried out to evaluate if the SmartFlares are indeed contained within endosomes and if the DMOG treatment changes the location of the SmartFlares (Figures 10-12).

![Co-localisation analysis of Cy5-Uptake Control (UC), Cy5-Scramble Control (SCR) and Cy3-VEGF (VEGF) SmartFlares with FITC-dextran with or without the presence of dimethyloxalylglycine (DMOG).](image)

**Figure 13.** Co-localisation analysis of Cy5-Uptake Control (UC), Cy5-Scramble Control (SCR) and Cy3-VEGF (VEGF) SmartFlares with FITC-dextran with or without the presence of dimethyloxalylglycine (DMOG).

The analysis shows that there is co-localisation occurring some more substantial in certain conditions. There appears to be a decrease in co-localisation after DMOG treatment for the Cy5-Uptake and Cy5-Scramble Control SmartFlares, whereas there is an increase in co-localisation for the Cy3-VEGF SmartFlare.
Again there is varied co-localisation of the SmartFlares and the lysosome marker with changes being seen after the DMOG treatment. The Cy3-VEGF SmartFlare with or without DMOG shows significant co-localisation with LAMP1 suggesting that it is contained within the endosomes.

**Figure 14.** Co-localisation analysis of Cy5-Uptake Control (UC), Cy5-Scramble Control (SCR) and Cy3-VEGF (VEGF) SmartFlares with anti-LAMP1 primary antibody (lysosome marker) with or without the presence of dimethyloxalylglycine (DMOG).

**Figure 15.** Co-localisation analysis of Cy5-Uptake Control (UC), Cy5-Scramble Control (SCR) and Cy3-VEGF (VEGF) SmartFlares with anti-Transferrin receptor primary antibody (recycling compartment marker) with or without the presence of dimethyloxalylglycine (DMOG).
The analysis shows before DMOG treatment there seems to be fairly similar co-localisation of all the SmartFlares with the TFN-R recycling compartment antibody marker. Consistent with the previous co-localisation analysis there is again a change in the co-localisation distribution after treatment with DMOG.

It should be noted again that the poor uptake seen with the Cy3-VEGF SmartFlares without DMOG (Figures 8A & 9A) is likely to have affected the co-localisation results for this condition.

5. Discussion

In this study we have used the commercially available oligonucleotide functionalised gold nanoparticles known as SmartFlare RNA detection probes to try and detect VEGF mRNA expression within live HeLa cells. These probes are sold as being able to enter cells without addition agents, are non-toxic to cells and are able to gain access to the cytosol to detect specific mRNA in live cells (Merk Millipore, 2015), however we have not found this to be the case in the experiments we have carried out.

As discussed earlier nanoparticles can be taken up into cells using various endocytic mechanisms, but there are many factors such as the size and surface chemistry of the nanoparticle bioconjugate that can affect its uptake (Nuri & Ji-Ho, 2014). For the SmartFlares it is surprising that they should easily be taken up into cells as the densely packed oligonucleotides creates a negative charge around the particle, which is usually unfavorable for uptake (Giljohann et al, 2007). This could partially explain why uptake only appeared to occur in a subset of cells for all of the experiments for example as seen in Figure 3. Another factor than could affect uptake of oligonucleotide functionalized gold nanoparticles was investigated by the Chad Mirkin group. They initially hypothesized that serum proteins may aid in cellular uptake but their research actually found that serum proteins reduced cellular
uptake when compared to the number taken up when serum free media was used (Pinal et al. 2010). It would be possible to see with transmission electron microscopy of pure SmartFlare samples and samples within medium if serum proteins are becoming associated. If it was found that the serum proteins are associating with the SmartFlares an experiment could be carried out using medium that doesn’t contain any serum proteins to see if this improves the uptake by the cells.

Another consistent observation seen throughout the experiments that raises many questions is the punctate signals for all three SmartFlares (Figures 5-7). The punctate nature of the signals, which suggests that the SmartFlares are contained within endosomes, is unexpected in the sense that SmartFlares are meant to escape these compartments, gain access the cytosol where if mRNA is detected a diffuse fluorescent signal is produced. To investigate if the SmartFlares are becoming entrapped within endosomes, we carried out immunostaining experiments. The antibodies selected were anti-LAMP1 to mark lysosomes and anti-transferrin receptor to mark the recycling compartments. The imaging results are shown in Figures 8-9 and these images were consistent with ones obtained previously with the live experiments as the cells show varied uptake of the SmartFlares and the signals are mostly punctate. To assess the co-localisation more quantitatively analysis was carried out using the JACoP plugin on ImageJ (Figures 13-15). It should be noted that unfortunately there was a problem with the Cy3-VEGF SmartFlare sample that was untreated with DMOG as there was poor signal for the FITC-dextran and SmartFlares. This could be due to the cells being left too long in unfavorable conditions during the experiment affecting uptake, or possibly fixation didn’t work properly leading to the cellular contents being released and washed away. These low levels of SmartFlare and FITC-dextran would have affected the corresponding co-localisation results and these experiments will need to be replicated. However the Cy5-Uptake and Cy5-Scramble Control SmartFlares showed similar co-
localisation with both LAMP1 and TFN-R suggesting they are contained within the endocytic system. Interestingly there is not complete co-localisation of the SmartFlares with FITC-dextran, which shows that they are not always found within the same compartments. This could suggest that they are being taken up separately from the FITC-dextran or there is possibly another mode of uptake causing them to be in different compartments. This problem of possible containment within endosomes has been investigated before by the Chad Merkin group where they used confocal immunofluorescence to characterize the subcellular localization of spherical nucleic acids (SNAs), which are the basis of SmartFlares. The results of this experiment actually concluded that the SNAs appear to be endocytosed and become sorted into early endosomes (Choi et al., 2013). The report goes on to conclude a success with their experiments stating that the SNAs provide a platform for various intracellular, in vivo application, but interestingly they do not discuss how the SNAs could escape endosomes which would be key for many intercellular uses (Choi et al., 2013).

An interesting observation was also seen for the three SmartFlares as they all showed similar signal intensities. Signal is expected for the Cy5-Uptake Control SmartFlare as it is designed to constantly fluoresce to show uptake of the SmartFlares. Also a signal can be expected for the Cy3-VEGF SmartFlares as these should fluoresce if mRNA is detected, but because of the punctate signal this suggests that VEGF mRNA is also contained within endosomes where hybridization should not take place. However a signal of similar intensity to the Cy5-Uptake Control and Cy3-VEGF SmartFlares was not anticipated for the Cy5-Scramble Control SmartFlares as these are designed to have no endogenous mRNA targets. This suggests that the oligonucleotides or reporter molecules are becoming cleaved or dissociating from the SmartFlares during the endocytic process.

DMOG treatment was carried out on cell samples of each SmartFlare. As DMOG is a PHD inhibitor which leads to the stabilization of HIF-1α it is expected to cause an increase in the
levels of VEGF mRNA, which should create an increased signal for the Cy3-VEGF SmartFlare. To confirm that DMOG treatment causes an increase in VEGF mRNA quantitative PCR was carried out. The results showed that a one-day treatment of 500 nM DMOG caused a 13-fold increase in VEGF mRNA compared to normoxic cells (Figure 2). A similar effect was expected with the Cy3-VEGF SmartFlares however this did not appear to be the case. Figures 10-12 show the effect of DMOG on each of the SmartFlares: there is a small increase in signal with the Cy3-VEGF SmartFlare but this could be due to the poor uptake of these cells so the result does need to be repeated. For the Cy5-Uptake and Cy5-Scramble Control DMOG treatment doesn’t appear to affect the signal intensity or distribution as they still show punctate signal in some cells. This result is expected, as the DMOG shouldn’t affect the controls, however with the Cy5-Scramble Control being a negative control its signal should have been lower than the other SmartFlares, but a similar signal is seen.

In conclusion, our experiences in using Merk Millipores SmartFlare RNA detection probes indicate that the probes do not working as advertised with all of our images showing varied uptake and punctate signal for the SmartFlares suggesting that they are not escaping endosomes. A very beneficial next step would be to investigate actually how these SmartFlares are being taken up and if endosomal processing is causing the punctate signals we have seen. It would also be interesting to see if the SmartFlares could be directly microinjected into the cells to see if they do then work as advertised, detecting the mRNA giving a diffuse signal.
References


