Published in final edited form as:

Anal Chem. 2008 October 1; 80(19): 7437–7444. doi:10.1021/ac8011347.

Assessing the Sensitivity of Commercially Available Fluorophores to the Intracellular Environment

ANTONY K. CHEN¹, ZHILIANG CHENG¹, MARK A. BEHLKE², and ANDREW TSOURKAS^{1,*}

¹*Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104*

²*Integrated DNA Technologies, Inc., Coralville, IA 52241*

Abstract

The use of fluorescence has become commonplace in the biological sciences, with many studies utilizing probes based on commercially available fluorophores to provide insight into cell function and behavior. As these imaging applications become more advanced it becomes increasingly important to acquire accurate quantitative measurements of the fluorescence signal. Absolute quantification of fluorescence, however, requires the fluorophores themselves to be insensitive to environmental factors such as non-specific protein interactions and pH. Here, we present a method for characterizing the sensitivity of fluorophores to the cytosolic environment by comparing their fluorescent intensity to an environment-insensitive reference signal before and after intracellular delivery. Results indicated that although the fluorescent intensity of a few fluorophores, e.g. fluorescein, were highly susceptible to the intracellular environment, other fluorophores, e.g. Dylight 649, Alexa647, and Alexa750, were insensitive to the intracellular environment. It was also observed that the sensitivity of the fluorophore could be dependent on the biomolecule to which it was attached. In addition to assessing the environmental sensitivity of fluorophores, a method for quantifying the amount of fluorophores within living cells is also introduced. Overall, the present study provides a means to select fluorophores for studies that require an absolute quantification of fluorescence in the intracellular environment.

INTRODUCTION

In recent years, commercially available fluorophores have become increasingly utilized in biological sciences for labeling and tracking biomolecules in situ, in cellulo, and even in living subjects. Applications have included the use of fluorescent probes to image protein expression, intracellular analytes¹⁻⁶, enzymatic activity (e.g. proteases)⁷⁻⁹, and even RNA expression¹⁰⁻¹⁴. As fluorescent imaging strategies have advanced, there has been a general trend towards more quantitative measurements of fluorescent signals, with the ultimate goal being absolute quantification. It is envisioned that the absolute quantification of fluorescence could allow the exact number of fluorophores within a compartment/cell to be quantified and correspondingly allow the number of target genes, proteins, or enzymes to be quantified. To date, however, one of the obstacles impeding absolute measurements of fluorescence is the inability to characterize the effect of environmental factors on the intensity of the fluorescent signal. Factors such as non-specific protein interactions and pH could have a dramatic effect on the fluorescence intensity of some fluorophores¹⁵. For example, it is widely known that the

*Address for correspondence: Andrew Tsourkas, Department of Bioengineering, University of Pennsylvania, 240 Skirkanich Hall, 210 S. 33rd Street, Philadelphia, PA 19104, atsourk@seas.upenn.edu, Phone: (215) 898-8167, Fax: (215) 573-2071.

Conflict of interest statement. I, Dr. Mark Behlke, am employed by Integrated DNA Technologies, Inc., (IDT) which offers oligonucleotides for sale similar to some of the compounds described in the manuscript. IDT is however not a publicly traded company and I personally do not own any shares/equity in IDT.

fluorescent intensity of fluorescein is highly susceptible to changes in pH and other environmental factors.

In attempt to eliminate the problems associated with non-specific interactions between the fluorescent indicator and intracellular contents, optical nanosensors have been developed that cage the reporter fluorophore within a protective matrix, which only permits the passage of ions but not cellular contents¹⁶⁻¹⁹. While this approach has proven effective for detecting small analytes, encapsulation of sensors in a protective matrix is generally not applicable for the purpose of detecting larger biomolecules (e.g. proteins and RNA), which often require direct interaction between the probe and the target. Therefore, if fluorescent probes are to be used to quantify the number of biomolecules, the fluorophores must be carefully chosen and assessed for their ability to provide accurate and reliable fluorescent signals. Unfortunately, methods for assessing the sensitivity of fluorophores to their environment is still lacking to date.

Here we present a novel method to assess the extent to which the emission of commercially available fluorophores is altered within the cytoplasm of single living cells. Specifically, changes in fluorescent emission were detected by comparing the fluorescent signal intensity of each fluorophore to that of a fluorescent reference probe that is insensitive to pH and shielded from the cytoplasmic environment. The fluorescent “reference” probe consisted of TMR-labeled dextran encapsulated within a liposome. Intracellular ratiometric measurements were compared with the fluorescence measurements acquired from analogous samples in buffer. All measurements were taken from images acquired directly on the microscope. In addition to presenting a methodology to assess the sensitivity of fluorophores to their environment, a method for quantifying the amount of reference dyes within living cells is also introduced.

EXPERIMENTAL SECTION

Liposome preparation

A lipid stock solution was prepared by combining 500 μ L of 10 mg/mL hydrogenated soy phosphatidylcholine (HSPC), 108 μ L of 20 mg/mL cholesterol, 55 μ L of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (18:0 PEG2PE) and 23.6 μ L of 0.25 mg/mL 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(Biotinyl (polyethylene glycol)-2000) (DSPE-PEG(2000) Biotin) (Avanti Polar Lipids, Inc.). This corresponds to a molar ratio of 66.5% HSPC, 28.5% cholesterol, 5% 18:0 PEG2PE, and 0.01% DSPE-PEG(2000) Biotin. The lipid stock solution was dried under nitrogen in a glass vial for 5 minutes and further dried under vacuum for at least 4 hours to ensure removal of all the chloroform. The sample was then reconstituted with 1 mL of 10 mg/mL FITC-labeled Dextran (MW 3000, Invitrogen) or TMR-labeled Dextran (MW 10,000, Invitrogen) in Phosphate Buffer (48 mM K_2HPO_4 , 4.5 mM KH_2PO_4 , 14 mM NaH_2PO_4), pH 7.2. Following incubation of the solution in a 55°C water bath for 1 hour, the mixture was subjected to five freeze-thaw-vortex cycles in liquid nitrogen and warm water (55°C), respectively. Next, the solution was extruded (21 times) through a 100-nm Nuclepore polycarbonate filter using a stainless steel extruder and then a 50-nm pore size polycarbonate membrane (Avanti Polar Lipids, Inc.). The extruded liposomes were separated from free dextran via size exclusion chromatography using Sepharose CL-4B (Sigma). The size distribution of the purified liposomes was assessed via dynamic light-scattering (DLS) using a Zetasizer Nano S (Malvern Instruments). The concentration of the fluorophores encapsulated within the liposomes was determined spectrophotometrically with a Cary100 spectrophotometer (Varian). All concentration measurements were adjusted to account for the liposome absorbance at the respective absorbance peak wavelengths of FITC or TMR.

Preparation of fluorescently-labeled NeutrAvidin

NeutrAvidin (Pierce) was dissolved in 50 mM Sodium Borate buffer, pH 8 at a concentration of 10 mg/mL and reacted with Texas Red-NHS ester (Invitrogen), Atto647-NHS ester (Sigma), Dylight 649-NHS ester (Pierce), Alexa647-NHS ester (Invitrogen), Cy5-NHS ester, Cy7-NHS ester (Amersham Biosciences) or Alexa750-NHS ester (Invitrogen) at dye to NeutrAvidin molar ratio of 2.5:1. Similarly, an additional sample of NeutrAvidin was labeled with FITC (Sigma) at a dye to NeutrAvidin molar ratio of 10:1. The fluorescent conjugates were purified on NAP-5 gel chromatography columns (Amersham Biosciences) in Phosphate Buffer. The number of fluorophores per NeutrAvidin was determined using a Cary100 spectrophotometer (Varian). It was determined that there were, 1.2 Atto647, 1.1 Dylight 649, 1.6 Alexa647, 1.5 Cy5, 1.5 Cy7, 1.7 Alexa750, and 3.6 FITC per NeutrAvidin, respectively.

Synthesis of molecular beacons and RNA targets

Antisense firefly luciferase molecular beacons (MB) possessing a TEX613 fluorophore (Texas Red derivative) or Cy5 fluorophore at the 5' end, an Iowa Black RQ quencher, IBRQ, at the 3' end, and a biotin-dT group incorporated in the 3' stem were synthesized by Integrated DNA Technologies, Inc (Coralville, IA, USA). The TEX613 antisense luciferase MB possessed a 2'-O-methyl RNA backbone with the sequence TEX613-mGmUmCmAmCmCmUmCmAmGmCmGmUmAmAmGmUmGmAmUmGmTmCmG (biotin-dT)mGmAmC-IBRQ. The Cy5 antisense luciferase MB possessed a DNA backbone with the sequence Cy5-GTCACCTCAGCGTAAGTGATGTCG(biotin-dT)GAC-IBRQ. A complementary luciferase RNA oligonucleotide target was also synthesized, with the sequence rUrGrGrArCrArUrCrArCrUrUrArCrGrCrUrGrArGrUrA.

Preparation of NeutrAvidin-liposome conjugates and molecular beacon-liposome conjugates

Fluorescently labeled NeutrAvidins were conjugated to the TMR-dextran-encapsulated liposomes via biotin-NeutrAvidin ligand binding chemistry. Specifically, each of the fluorescently labeled NeutrAvidins described above were diluted to 25 μ M and 30 μ L of each sample was incubated with 100 μ L of the biotinylated liposomes at room temperature overnight. The NeutrAvidin-Liposome conjugates were then purified on Superdex columns (Amersham Bioscience) in Phosphate Buffer and concentrated on Microcon centrifugal filter devices (YM-50, Millipore).

Similarly, to synthesize molecular beacon (MB)-liposome conjugates, 100 μ L of biotinylated liposomes were reacted with 30 μ L of 25 μ M unlabeled NeutrAvidin. The NeutrAvidin-Liposome conjugates were then purified on Superdex columns in Phosphate Buffer and concentrated on Microcon centrifugal filter devices (YM-50, Millipore). Then, 4 μ L of 100 μ M MBs were added to the 100 μ L of the purified NeutrAvidin-LP conjugates. The resulting MB-liposome conjugates were purified from the unbound MBs on Superdex columns and concentrated on YM-50 filter devices. The MBs were subsequently hybridized by incubating overnight with complementary RNA targets (4 μ L of the 100 μ M RNA) at room temperature.

pH sensitivity assay

Each fluorescently labeled NeutrAvidin conjugate was diluted to 250 nM in buffers with pH values ranging from 3 to 10. The specific buffers utilized were 0.1 M Sodium Citrate Buffer for pH 3, 4, 5, and 6, 0.1 M Sodium Phosphate Buffer for pH 6, 7, and 8, and 0.1 M Glycine-NaOH Buffer for pH 9 and 10. The fluorescent intensity of each sample was measured with a SPEX FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon). Excitation and emission wavelengths were adjusted according the reported maximum for each fluorophore. Analogous

studies were performed with 50 nM of FITC-Dextran and TMR-Dextran, free and encapsulated within liposomes.

Fluorescence quenching assay

Samples containing 50 nM of free FITC-dextran or 50 nM FITC-dextran encapsulated within liposomes were prepared in Phosphate Buffer containing 1 mg/mL BSA. Analogous samples were also prepared with 0.1 mg/mL anti-fluorescein antibodies (Invitrogen). Fluorescent intensity measurements were acquired with a SPEX FluoroMax-3 spectrofluorometer using a single excitation at 490 nm. The fluorescent emission was collected from 500 nm to 800 nm.

Preparation of fluorescent water-in-oil emulsions

The procedure used to prepare water-in-oil emulsions was modified slightly from Pietrini et al.²⁰. Briefly, Span 80 (447 mg) and Tween 80 (54 mg) were added to mineral oil (24 g) and the mixture was vortexed vigorously. A 3 mL aliquot of this mixture was then added to a glass vial and stirred. An aqueous sample (10 μ L) containing the fluorescent conjugate of interest was then added dropwise to form a microemulsion. Each emulsion was mixed for two minutes. An aliquot of the emulsion sample was then placed on the coverslip for microscopic analysis.

Cell Culture

MCF-7 breast cancer cells (ATCC, Manassas, VA, USA) were cultured in minimum essential media (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1mM sodium pyruvate and 10% fetal bovine serum (FBS). The cells were incubated in 5% CO₂ at 37°C. For all live-cell imaging experiments, the MCF-7 cells were seeded on a 60 mm Petri dish at a confluency of 10-30% in Dulbecco's MEM media without phenol red supplemented with 10% FBS.

Fluorescent imaging

All microscopy images were acquired with an Olympus IX81 motorized inverted fluorescence microscope equipped with a back-illuminated EMCCD camera (Andor), an X-cite 120 excitation source (EXFO), and Sutter excitation and emission filter wheels. Images of the FITC-Dextran and TMR-Dextran encapsulated liposomes were acquired using the filter sets (HQ480/40, HQ535/50, Q505LP) and (HQ545/30, HQ610/75, Q570LP), respectively. Images of the Texas-Red labeled NeutrAvidins were acquired using the filter set (HQ560/55, HQ645/75, Q595LP). Cy5, Alexa647, Dylight 649 and Atto647-labeled NeutrAvidins were acquired using the filter sets (HQ620/60, HQ700/75, Q660LP). Images of the Alexa750 and Cy7 were acquired using the filter set (HQ710/75, HQ810/90, Q750LP). All filter sets were purchased from Chroma. A LUC PLAN FLN 40x objective (NA 0.6) was used for all cell imaging studies. Results were analyzed with NIH ImageJ.

Standardization curves for quantitative microscopic analysis

Aqueous samples containing 1, 2.5 or 5 μ M TMR-dextran encapsulated liposomes in Phosphate Buffer containing 1 mg/mL BSA were injected into paraffin oil in a Mattek glass-bottom dish using an FemtoJet and Injectman NI 2 (Eppendorf) microinjection system creating water-in-oil bubbles. Water-in-oil bubbles (i.e. microemulsions) were also made by slowly adding TMR-dextran encapsulated liposomes into a stirring mixture of Span 80, Tween 80, and mineral oil as described above. To compare the two methods, fluorescent images of the resulting water-in-oil bubbles were acquired at the camera setting of 1 $\times$ 1 binning with a 20x objective (NA 0.4). At least 10 bubbles were acquired for each TMR-dextran concentration. The diameter of each bubble was measured with IPLabs and the volume was calculated assuming a spherical geometry. The number of TMR molecules in each bubble was determined by multiplying the volume of each bubble by the concentration of TMR. The background subtracted fluorescence

of each bubble was determined using NIH ImageJ. Specifically, a region of interest (ROI) was drawn around each bubble in the TMR image and the total fluorescent intensity was measured using ImageJ. Similarly, the total fluorescence intensity from an equal sized ROI that was drawn around a “background” region was also measured. Standardization curves were then constructed to correlate the total background subtracted fluorescence to the number of TMR molecules within each bubble.

To determine whether microinjection or emulsification causes a disruption in the liposome membrane, leading to the release of contents, bubbles containing FITC-dextran encapsulated liposomes (2.5 μ M) were mixed with anti-FITC antibodies at 0.7 mg/mL prior to microinjection and emulsification. The fluorescence intensity of the bubbles (normalized by volume) was then compared to water-in-oil bubbles containing FITC-dextran encapsulated liposomes in the absence of anti-FITC antibodies. Further comparisons were made with water-in-oil bubbles containing free FITC-dextran in the presence and absence of anti-FITC antibodies at the same concentrations.

Ratiometric Analysis

Emulsions containing NeutrAvidin-liposome conjugates were prepared as described above. For each bubble, two images were acquired, one corresponding to the NeutrAvidin labeled with the commercial fluorophore under investigation and the other to the TMR- or FITC-dextran encapsulated liposome, i.e. “reference” fluorophore. A region of interest (ROI) was drawn around each bubble and the total fluorescent intensity was measured in each image. Similarly, the total fluorescence intensity from an equal sized ROI drawn around a “background” region was also measured for each image. The background subtracted fluorescence measurement for each commercial fluorophore, Fc, and reference fluorophore, Fr, was then calculated.

To account for the signal bleed-through from the reference fluorophore into the commercial fluorophore channel, emulsions containing only the liposomes (i.e. reference fluorophore) were prepared and fluorescent images were acquired in the reference channel and in each of the respective commercial fluorophore channels. The fluorescent ratio comparing the fluorescence of the liposome in the commercial fluorophore channel to that in the reference channel was used for subsequent spectral unmixing calculations^{21, 22}. At least 10 bubbles were analyzed for each liposome sample.

Ratiometric analysis was performed on images of MCF-7 cells microinjected with the NeutrAvidin-liposome conjugates using a method analogous to that used with emulsions, with an additional step to subtract autofluorescence from the cells. Autofluorescence images of the cell were acquired in the channels corresponding to the commercial and “reference” fluorophores, respectively, prior to microinjection. The background-subtracted autofluorescence signals were then used to correct the respective background-subtracted commercial fluorophore and “reference” signals following microinjection. At least 10 cells were analyzed for each conjugate. It should be noted that the reference-commercial fluorophore pair were chosen to minimize spectral overlap. Also, far-red shifted dyes were chosen because cells emit negligible levels of autofluorescence at those wavelengths.

RESULTS AND DISCUSSION

Most commercially available fluorophores are insensitive to pH

It has been established that the pH within the intracellular environment can be as low as 4.5 in lysosomes and as high as 8.0 in the mitochondria²³. Therefore, it can be argued that if any fluorophore is to be universally utilized for quantitative fluorescent measurements in living

cells, it should be insensitive to pH over this range. Accordingly, we tested the effect of pH on the fluorescence intensity of nine different commercially available fluorophores that had been conjugated to NeutrAvidin (Figure S-1). It was found that fluorescence intensity of Cy5-, Cy7-Texas Red- and Dylight 649-NeutrAvidin conjugates as well as TMR-dextran was generally insensitive to pH over a range of 3 to 10. Alexa647- and Alexa750-NeutrAvidin conjugates were insensitive to pH from 5 to 10, but exhibited a ~18% decrease in fluorescence intensity at pH 3 and 4. Atto647 was insensitive to pH over a range of 4 to 10, but exhibited an increase in fluorescence at pH 3. As expected, fluorescein, i.e. FITC-dextran, proved to be the most sensitive to pH, exhibiting more than a 95% decrease in fluorescence intensity as the pH was decreased from 10 to 3.

Lipid bilayer provides protective barrier for encapsulated TMR-dextran

Since TMR-dextran had limited spectral overlap with most of the fluorophores tested, and because of its insensitivity to pH we chose to use TMR-dextran as a 'reference' fluorophore for which to compare the intensity of the other fluorophores in living cells. Of course, if TMR-dextran were to be a suitable reference fluorophore it must itself be insensitive to all environmental factors not just pH. If this condition is met, then any change in the ratio comparing the fluorescence intensity of other commercial available fluorophores to that of TMR-dextran would indicate their sensitivity to the intracellular environment. Of course, the potential for non-specific interactions between TMR-dextran and intracellular biomolecules (e.g. proteins, lipids, enzymes, and nucleic acids) was a major concern. Thus, to ensure that the fluorescence intensity of the TMR-dextran was not altered by any species within the intracellular environment, it was encapsulated within liposomes. It was hypothesized that liposomes would shield the encapsulated TMR-dextran from biomolecules and analytes that may affect its fluorescence.

To validate that liposomal membranes could effectively protect encapsulated fluorophores from surrounding biomolecules, an antibody quenching assay was adopted²⁴. Specifically, water-soluble, FITC-dextran was encapsulated within liposomes and mixed with anti-fluorescein antibodies. Spectrofluorometric measurements revealed that when FITC-dextran was encapsulated within liposomes the fluorescent signal remained unquenched even in the presence of the anti-fluorescein antibodies (Figure S-2). Conversely, the anti-fluorescein antibodies quenched the fluorescent signal of free FITC-dextran by more than an 85% compared with free FITC-dextran in the absence of antibody. These results indicated that liposomal entrapment could be used as an effective mechanism to protect fluorophores from protein interactions.

Interestingly, when an antibody-quenching assay was performed with TMR-dextran, it was found that even free TMR-dextran was not effectively quenched by anti-TMR antibodies (~2% quenching) (Figure S-3A). Presumably, the presence of the 10 kDa dextran sterically hindered the access of anti-TMR antibodies to TMR. This was supported by measurements showing ~57% quenching of fluorescence when unconjugated TMR dyes were incubated with anti-TMR antibodies (Figure S-3B). Overall, these findings demonstrate that even when proteins have a specific affinity for TMR-dextran, they have little effect on TMR fluorescence. Therefore, it may be speculated that non-specific binding would have even less of an effect on TMR fluorescence. Nonetheless, TMR-dextran was encapsulated within liposomes for additional protection. As expected, encapsulation of the TMR-Dextran into liposomes effectively protected the dye from being quenched by anti-TMR antibodies.

To investigate the permeability of liposomes to small analytes, liposomes were prepared with pH-sensitive FITC-dextran encapsulated within the core. These FITC-encapsulated liposomes were then suspended in buffers with pH values ranging from 3 to 8. The intensity of the FITC fluorescence signal did not exhibit any significant changes from pH 3 to 7, which is in sharp

contrast to free FITC-dextran in the same buffers (Figure 1A). As the pH increased from 7 to 8 only a 10% increase in fluorescence intensity was observed. All measurements were made ~30 minutes after preparation of the sample. This suggests that even H⁺ ions are inefficient at traversing the liposomal membrane and further implies that small analytes that may act as quenching species would likely not be able to traverse the liposomal membrane. As expected, the TMR-dextran encapsulated liposomes were insensitive to pH from 3 to 8 (Figure 1B). It should also be noted that in addition to pH, TMR-dextran is believed to be insensitive to ionic compounds. Three different ionic buffers were used to span the pH range from 3 to 10, but none influenced the fluorescence intensity of TMR. Combined, these results suggest that the fluorescent intensity of TMR-encapsulated liposomes would not be altered in the intracellular environment and could thus serve as an effective reference marker.

Water-in-oil microinjections allow for quantitative microscopy measurements of fluorescent samples

As mentioned above, it was hypothesized that the sensitivity of various commercially available fluorophores to the intracellular environment could be evaluated by comparing their fluorescent signal (Fc) to that of the 'reference' fluorophore (Fr), i.e. TMR-dextran encapsulated liposomes, before and after intracellular delivery. To ensure the relative amount of each dye remained constant following intracellular delivery, the commercially available fluorophores were conjugated to NeutrAvidin and linked to the reference liposomes via a biotin-NeutrAvidin linkage. Further, to acquire a baseline measurement of the fluorescent ratio Fc/Fr in vitro to which live cell measurements would be compared, a methodology was adopted that involved microinjecting a known concentration of the fluorescent conjugates into oil²⁵. Images of the fluorescent bubbles were acquired directly on the microscope using the same image acquisition parameters as were used for live cell studies. This was necessary to maintain consistency between in vitro and live cell measurements. Example images of a fluorescent water-in-oil bubble are shown in Figure S-4. The background subtracted fluorescence intensity of each bubble in both the reference channel (Fr) and the commercial fluorophore channel (Fc) could easily be determined and the ratio Fc/Fr calculated.

Microinjection does not cause a disruption of liposomes

One concern with using microinjection for delivering liposomal conjugates into oil and live cells was that this procedure could cause a disruption in the liposomal membrane, and a corresponding leakage of TMR-dextran from its protected environment. To examine whether microinjection created any disruptions in the liposome membranes, an antibody-based quenching assay was performed. Specifically, FITC-dextran encapsulated liposomes were mixed with anti-fluorescein antibodies prior to microinjection into oil. Based on preliminary water-in-oil measurements, binding of free FITC-dextran by these antibodies caused more than a 81% decrease in fluorescence intensity (Figure S-5); therefore, if any FITC-dextran was released from the liposomes during microinjection into oil its fluorescent signal would be quenched. Fortunately, no loss of FITC-dextran fluorescence was observed following microinjection (Figure S-5).

As further proof that the liposomes were not destroyed during microinjection, it was readily observed that the TMR-dextran encapsulated within liposomes was strictly confined to the cytoplasm (Figure 2). Conversely, free TMR-dextran (10 kDa) was small enough to pass through the nuclear pores, generating a bright fluorescent signal in the nucleus (data not shown).

Sensitivity of commercial fluorophores to the cytosolic environment

To determine the sensitivity of commercial fluorophores to the intracellular environment, the signal intensity of the fluorescently-labeled NeutrAvidins (Fc) that were conjugated to the liposome surface was compared with the fluorescence intensity of the TMR-dextran (Fr)

encapsulated within the liposomes. The fluorescence ratio F_c/F_r was determined for each dye using the water-in-oil approach described above and following microinjection into the cytoplasm of live cells. An example image of a cell following microinjection is shown in Figure 2. It is evident that both the commercial dye and the TMR-dextran encapsulated liposomes co-localized within the cytoplasm of the cell. All ratiometric calculations were normalized against the ratio obtained from the water-in-oil studies. The normalized ratiometric data for each of the fluorophores examined in live cells is shown in Figure 3.

Of all the fluorophores tested, it was not surprising to find that FITC was the most sensitive to the intracellular environment, exhibiting a 48% enhancement in fluorescence intensity following microinjection into live cells ($p < 0.05$, two-tailed t-test, unpaired). The next largest shift in fluorescence was seen with Atto647, which exhibited a 31% enhancement in signal following microinjection into cells ($p < 0.05$). Texas Red exhibited a 14% enhancement in fluorescence ($p < 0.05$) in cells and Cy7 exhibited a 19% enhancement ($p < 0.05$). All of the other fluorophores exhibited less than a 10% change in fluorescence following intracellular delivery. Specifically, Cy5 exhibited a 7% enhancement of fluorescence ($p < 0.05$) in the intracellular environment and no statistically significant change in fluorescence intensity was observed for Dylight 649 ($p=0.15$), Alexa647 ($p=0.533$), or Alexa750 ($p = 0.08$).

Sensitivity of molecular beacons to the cytosolic environment

In addition to testing the sensitivity of fluorophore-NeutrAvidin conjugates to the intracellular environment, we also investigated the sensitivity of molecular beacons (MBs) to the cytosol. MBs are dual-labeled oligonucleotide probes that are labeled at one end with a fluorophore and at the other end with a quencher. In the absence of complementary nucleic acid targets MBs form a stem loop structure, which forces the fluorophore and quencher into close proximity. As a result, the fluorescent signal is efficiently quenched. Upon hybridization, the fluorophore and quencher are separated and fluorescence is restored. The unique ability of MBs to generate a substantial enhancement in fluorescence upon hybridization has led to their increasing use as a probe for imaging RNA in live cells¹⁰⁻¹⁴. To date, MBs have been strictly used to qualitatively measure the presence of RNA; however, if the fluorescent signal could be accurately quantified without interference from the surrounding environment, then absolute quantification of gene expression may be possible.

Two different molecular beacons were tested, Cy5-MBs and Texas Red-MBs. Cy5-MBs and Texas-Red MBs were attached to the TMR-dextran encapsulated liposomes and FITC-dextran encapsulated liposomes, respectively, via a biotin-NeutrAvidin linkage. Further, the MBs were hybridized to complementary RNA targets prior to injection so the fluorescent signal would not be quenched. Interestingly, neither MB conjugate exhibited a statistically significant sensitivity to the intracellular environment (Figure 4).

Water-in-oil Microinjection and microemulsion techniques can be used to generate fluorescent microscopy standardization curves

The ability to acquire fluorescent microscopy images of water-in-oil bubbles containing a known concentration of fluorophore provides a simple methodology for defining the relationship between total fluorescence intensity and fluorophore number. The number of fluorophores within each water-in-oil bubble is easily determined since the concentration of fluorophore is known and the volume of the bubble is easily calculated (assuming a spherical geometry). Fluorophore number (and total fluorescence intensity) was quantified over several orders of magnitude by varying the concentration of the fluorescent dye and by forming bubbles of varying diameter. As expected, the total fluorescence intensity increases linearly with increasing numbers of fluorophores (Figure 5A).

While microinjecting fluorescent samples into oil is one possible method for drawing a relationship between fluorescence intensity and fluorophore number, it can be tedious, inefficient, and it requires specialized equipment. Therefore, we evaluated whether water-in-oil emulsions could also be used to acquire the same results. Water-in-oil emulsions are extremely simple to prepare, do not require specialized equipment, and allow for the formation of many bubbles at a time. Further, there is some precedent for using emulsions to efficiently encapsulate small aqueous samples. For example, water-in-oil emulsions have previously been used for in vitro compartmentalization to host biological reactions, including in-vitro transcription and synthesis of enzymes^{26, 27} and protein molecules²⁰. To determine whether emulsions could also be used to determine the relationship between fluorescence intensity and fluorophore number, emulsions were prepared with known concentrations of fluorophore and the relationship between total fluorescence intensity and fluorophore number were compared with analogous microinjection experiments. As shown in Figure 5, both microinjection and emulsion experiments yielded a very similar linear correlation between fluorescence intensity and the number of fluorescent molecules. Specifically, the slopes were 0.026 fluorescent units/TMR molecule for microinjection and 0.027 fluorescent units/TMR molecules for emulsion, with both correlation coefficients being > 0.99 . These results suggest that water-in-oil emulsion and microinjection methods can be used interchangeably.

If the fluorophore under investigation is insensitive to the intracellular environment, the correlation between total fluorescence intensity and fluorophore number can serve as a “standardization curve” for quantifying the absolute number of fluorophores within single living cells. A fluorescent microscopy standardization curve was established using the TMR-dextran encapsulated liposomes under the 40x objective, with a camera setting of 2×2 binning, the same settings as used to acquire the cell images. A linear correlation between the total fluorescent intensity and the number of TMR molecules within each bubble was determined to be 1.3 AU/molecule with a correlation coefficient > 0.99 (data not shown). As expected, the sensitivity of detection was much higher with an objective with higher magnification and higher camera binning settings. Comparing the reference signals in the cells injected with TMR-dextran encapsulated liposomes with the pre-established standardization curve, the amount of the reference dyes delivered into the cells were determined to vary significantly from cell-to-cell, ranging from as low as 302,918 copies to as high as 21,790,259 copies of TMR molecules in this study (using a single injection concentration). The concentration of TMR-dextran used for this study was kept high to ensure that the fluorescent signal was above the autofluorescence of the surrounding media and cell. However, it should be noted that even for water-in-oil emulsions, autofluorescence of the surrounding media limited the lower detection limit to $\sim 15,000$ TMR molecules. This lower detection limit can be improved by using dyes that emit in the far red. In the case of Cy5-NeutrAvidin, the lower detection limit was found to be ~ 500 molecules (Figure S-6). The lower detection limit is also likely to be improved in special cases where the dyes inside a living cell are highly localized. Other parameters that can be adjusted to improve sensitivity include the excitation source, filter sets, objective, camera, and image acquisition settings.

In principle, the method for quantifying the amount of intracellular fluorescent molecules can be extended to molecular imaging probes, whereby the fluorescent signal corresponds to a specific number of biomolecular targets (e.g. RNA). Of course, this requires that the dyes themselves be insensitive to the intracellular environment and a relationship between the probe intensity and number of targets could be determined. In an alternative application, our results provide a means to monitor transfection efficiency of fluorescent probes into living cells. This may be important when it is necessary to normalize against cell-to-cell variations in fluorescence due to unequal transfection²⁸.

CONCLUSIONS

When fluorescent probes are designed to detect intracellular biomolecules, they should only report signal upon target recognition, with minimal influence from the environment. The present study describes a method to assess the sensitivity of commercially available fluorophores in the cytoplasmic environment. By comparing the fluorescence intensity of each fluorophore with that of a reference signal in solution and in live cells, we were able to quantify the absolute change in fluorescence intensity for each fluorophore in response to cytoplasmic interactions. A calibration curve correlating the total fluorescence intensity and the number of the reference dye molecules was also introduced. This curve can be used to determine the amount of probes introduced into the cells based on the intensity of the reference signal within cells. In principle, the method of external calibration can be extended to any dyes that are insensitive to the cellular environment. Altogether, these results can potentially benefit the design and optimization of molecular imaging probes for quantifying specific intracellular species including analytes, proteins, and RNA inside living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This material is based upon work supported in part by the National Institute of Health (NCI) R21 CA116102, the National Science Foundation BES-0616031, and the American Cancer Society RSG-07-005-01.

References

1. Iribe G, Kohl P. *Prog Biophys Mol Biol*. 2008
2. Mason, W. *Biological Techniques*. Academic Press; San Diego: 1993.
3. Nuccitelli, R. *Methods in Cell Biology*. Academic Press; San Diego: 1994.
4. Slavík J. *J Lumin* 1997;72–74. 575–577.
5. Srivastava A, Krishnamoorthy G. *Anal Biochem* 1997;249:140–146. [PubMed: 9212865]
6. Tour O, Adams SR, Kerr RA, Meijer RM, Sejnowski TJ, Tsien RW, Tsien RY. *Nat Chem Biol* 2007;3:423–431. [PubMed: 17572670]
7. Jaffer FA, Kim DE, Quinti L, Tung CH, Aikawa E, Pande AN, Kohler RH, Shi GP, Libby P, Weissleder R. *Circulation* 2007;115:2292–2298. [PubMed: 17420353]
8. Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, Tsien RY. *Proc Natl Acad Sci U S A* 2004;101:17867–17872. [PubMed: 15601762]
9. Law B, Weissleder R, Tung CH. *Bioconjug Chem* 2007;18:1701–1704. [PubMed: 17915958]
10. Bratu DP, Cha BJ, Mhlana MM, Kramer FR, Tyagi S. *Proc Natl Acad Sci U S A* 2003;100:13308–13313. [PubMed: 14583593]
11. Chen AK, Behlke MA, Tsourkas A. *Nucleic Acids Res* 2007;35:e105. [PubMed: 17702767]
12. Perlette J, Tan W. *Anal Chem* 2001;73:5544–5550. [PubMed: 11816586]
13. Santangelo PJ, Nix B, Tsourkas A, Bao G. *Nucleic Acids Res* 2004;32:e57. [PubMed: 15084672]
14. Tyagi S, Alsmadi O. *Biophys J* 2004;87:4153–4162. [PubMed: 15377515]
15. Graber ML, DiLillo DC, Friedman BL, Pastoriza-Munoz E. *Anal Biochem* 1986;156:202–212. [PubMed: 3740410]
16. Barker SL, Thorsrud BA, Kopelman R. *Anal Chem* 1998;70:100–104. [PubMed: 9435469]
17. Clark HA, Hoyer M, Philbert MA, Kopelman R. *Anal Chem* 1999;71:4831–4836. [PubMed: 10565274]
18. Healey BG, Li L, Walt DR. *Biosens Bioelectron* 1997;12:521–529. [PubMed: 9253155]
19. Panova AA, Pantano P, Walt DR. *Anal Chem* 1997;69:1635–1641. [PubMed: 9109355]

20. Pietrini AV, Luisi PL. *Chembiochem* 2004;5:1055–1062. [PubMed: 15300827]
21. Castleman KR. *Bioimaging* 1994;2:160–162.
22. Kato N, Pontier D, Lam E. *Plant Physiol* 2002;129:931–942. [PubMed: 12114548]
23. Asokan A, Cho MJ. *J Pharm Sci* 2002;91:903–913. [PubMed: 11948528]
24. McNamara KP, Nguyen T, Dumitrascu G, Ji J, Rosenzweig N, Rosenzweig Z. *Anal Chem* 2001;73:3240–3246. [PubMed: 11476221]
25. Lee GM, Thornthwaite JT, Rasch EM. *Anal Biochem* 1984;137:221–226. [PubMed: 6731800]
26. Griffiths AD, Tawfik DS. *EMBO J* 2003;22:24–35. [PubMed: 12505981]
27. Tawfik DS, Griffiths AD. *Nat Biotechnol* 1998;16:652–656. [PubMed: 9661199]
28. Dandekar DH, Kumar M, Ladha JS, Ganesh KN, Mitra D. *Anal Biochem* 2005;342:341–344. [PubMed: 15989929]

Figure 1.

Sensitivity of FITC-dextran and TMR-dextran encapsulated liposomes to pH. Maximum fluorescence intensity of solutions containing (A) 50 nM FITC-labeled dextran or 50 nM FITC-labeled dextran encapsulated in phospholipids suspended in buffers with pHs ranging from 3 to 8. (B) 50 nM TMR-labeled dextran or 50 nM TMR-labeled dextran encapsulated in phospholipids suspended in buffers with pH ranging from 3 to 8.

Figure 2.

Fluorescent images of single cells injected with fluorescent NeutrAvidin-liposome conjugates. Commercial fluorophores conjugated to liposomes containing TMR-dextran were microinjected into MCF-7 cells. Images of the commercial fluorophore (left) and TMR-dextran (middle) were acquired. A ratiometric image (right) was obtained by dividing the background-subtracted reporter image by the background-subtracted reference image.

Figure 3.

Sensitivity of commercially available fluorophores to the intracellular environment. The fluorescence ratios comparing the signal intensity of various fluorescently labeled NeutrAvidins (Fc) to TMR-dextran (Fr) encapsulated within liposomes were determined for samples injected into oil, creating bubbles, and into living MCF-7 cells. All ratiometric calculations, Fc/Fr, were normalized against the ratio obtained from the water-in-oil studies. At least 10 cells or 10 bubbles were analyzed for each dye-labeled liposome conjugate. The error bars represent the standard error of the mean. The * represents statistical significance ($p < 0.05$, two-tailed t-test, unpaired) between water-in-oil and live cell measurements.

Figure 4.

Sensitivity of molecular beacon fluorescence to the intracellular environment. Prehybridized Cy5- or Texas Red-labeled molecular beacons were conjugated to liposomes containing TMR-dextran and microinjected into oil, creating bubbles, and into living MCF-7 cells. The ratio comparing the molecular beacon signal (Fc) to the TMR signal (Fr) was calculated. All ratiometric calculations, Fc/Fr, were normalized against the ratio obtained from the water-in-oil studies. At least 10 cells or 10 bubbles were analyzed for each molecular beacon-labeled liposome conjugate. The error bars represent the standard error of the mean. The * represents statistical significance ($p < 0.05$, two-tailed t-test, unpaired) between water-in-oil and live cell measurements.

Figure 5.

Standardization curves correlating fluorescence intensity to the number of TMR molecules. Standardization curves were generated by analyzing fluorescent bubbles that were formed by either (A) injecting liposome encapsulated TMR-dextran into oil or (B) by making a microemulsion with the same sample. A linear, positive correlation between fluorescence intensity and number of TMR molecules was observed for both the (A) injection and (B) emulsion techniques. At least 30 bubbles were analyzed for each method.