PBS® A New Ultrastable Probe for BioMedical Research and Imaging

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Introduction – The Problem

Fluorescent and phosphorescent probes, such as fluorescent proteins, quantum dots, small fluorescent molecules, and small phosphorescent molecules have revolutionized the fields of biomedical research, molecular biology, and biophysics; additionally, they have had significant impacts in medical diagnostics. The ability to link these probes to a molecule of interest has allowed for the use of many important techniques, such as fluorescence microscopy, immunocytochemistry, immunohistochemistry, fluorescence *in situ* hybridization, flow cytometry, and fluorescence-linked immunosorbent assays. These probes have also made super resolution imaging and single-molecule microscopy possible, offering insight into the inner workings of cells and normal/altered biomolecular interactions.

There are various advantages offered by each class of fluorescent and phosphorescent probes. *Fluorescent proteins*, such as green fluorescent protein (GFP), have large molar absorptivities and high quantum yields, resulting in probes with high luminosity. Fluorescent proteins can be genetically fused or chemically conjugated to a protein of interest, though conjugation of GFP with antibodies has seldom been explored. *Small fluorescent molecules*, frequently referred to as fluorescent dyes, likewise demonstrate high luminosity due to their large molar absorptivity and quantum yields. Fluorescent dyes can be synthesized to have reactive groups, such as succinimidyl esters and maleimides, allowing them to be chemically conjugated to a molecule of interest. The small size of fluorescent dyes compared to labeled biomolecules (300-500 Da compared to 10,000-100,000 Da proteins) generally prevents the fluorescent dyes from interfering with the function of the proteins that they label. *Small phosphorescent molecules* can likewise be synthesized to contain reactive groups, allowing them to be easily linked to target molecules. The luminescence lifetimes of phosphorescent molecules are significantly longer than those of fluorescent molecules, making it easy to isolate their signals through the use of time-gated spectroscopic techniques (*vide infra*). *Quantum dots* have high luminosities, again resulting from the combination of large molar absorptivity and high quantum yields. Quantum dots can be functionalized with the same reactive groups as fluorescent dyes, allowing them to be chemically conjugated to molecules of interest.

While the probes described above have enabled scientists to perform great work, they possess certain intrinsic problems. Both fluorescent proteins and fluorescent dyes are susceptible to photoblinking (the intermittent loss of photon emission), and photobleaching (a chemical change that results in a total loss of fluorescence) (1,2). Fluorescent proteins can also misfold during expression or denature during an experiment, resulting in a complete loss of fluorescence. While quantum dots photobleach slowly, they undergo a process called photobluing, whereby the nanoparticles eject atoms into the surrounding solution, changing the photophysical properties of the probes. As a result, both the absorption and emission properties of the nanoparticles

undergo a blue shift before they eventually bleach (3,4). The dissolution of quantum dots during the bluing process is particularly problematic for living cells as quantum dots are frequently composed of heavy metals, such as cadmium, which are toxic to cells. Passage of quantum dots through a cell membrane without harming or killing the cell can also be quite challenging; furthermore, conjugation of quantum dots to biomolecules of interest is significantly more difficult than the conjugation of dyes or making a fusion protein with GFP. Due to their relatively large sizes and the presence of multiple reactive groups on their surface, conjugatable quantum dots can bind to multiple copies of the target biomolecule, resulting in aggregation that can disrupt cellular function when they are being used for *in vivo* imaging (3).

Additionally, autofluorescence, or the fluorescence of endogenous biomolecules further decreases the sensitivity of these measurements by increasing the signal of the background. While the background from autofluorescence can be somewhat mitigated using fluorescence lifetime imaging (FLIM), the fluorescence lifetimes of most commonly used fluorophores (Table 1) are unfortunately in the same range of the fluorescence lifetimes of the background. Distinguishing between these lifetimes in FLIM measurements is possible; however, such analysis is time-consuming, technically difficult, and often not quantitatively definitive. While the longer luminescence lifetimes of phosphorescent molecules makes it easier to separate the signal of the probe molecule from the autofluorescent signal, these molecules still photobleach and their luminosities are significantly lower than those of fluorescent proteins, fluorescent dyes, and quantum dots.

Table 1. List of common fluorescent probes and endogenous fluorophores and their fluorescence lifetimes.

Fluorophore	Lifetime (ns)
GFP (5)	2.40
Atto 655 (6)	1.87
Alexa Fluor 532 (7)	2.53
Alexa Fluor 546 (7)	4.06
Cy 5 (6)	0.91
Rhodamine 6G (8)	4.0
NADPH (9)	0.3-2.3
Riboflavin (9)	4.12
FAD (9)	2.91
Melanin (9)	0.1-8

The Solution – PBS® A New Luminescent Bio-Nanoparticle

Of the problems listed above, the most detrimental to scientific research and medical diagnostics is the lack of photostability, which greatly limits their imaging time to only a few minutes, thereby lowering the sensitivity and reliability of quantitative measurements. This is particularly a problem for trace (picomolar) and ultratrace (femtomolar) detection. Through our collaboration with Washington State University, Photon Biosciences, LLC has developed PBS®, a new ultrastable, ultrasensitive phosphorescent probe. PBS® probe technology combines a genetically engineered protein with a proprietary biomineralization process, resulting in a phosphorescent bionanoparticle. Unlike phosphorescent and fluorescent probes currently on the market, PBS® probes never photobleach, a trait that will allow researchers to perform experiments that are currently either difficult or impossible. In this paper, we will discuss some of the properties and applications of PBS® probes.

Spectroscopic Properties and Spectral Tunability

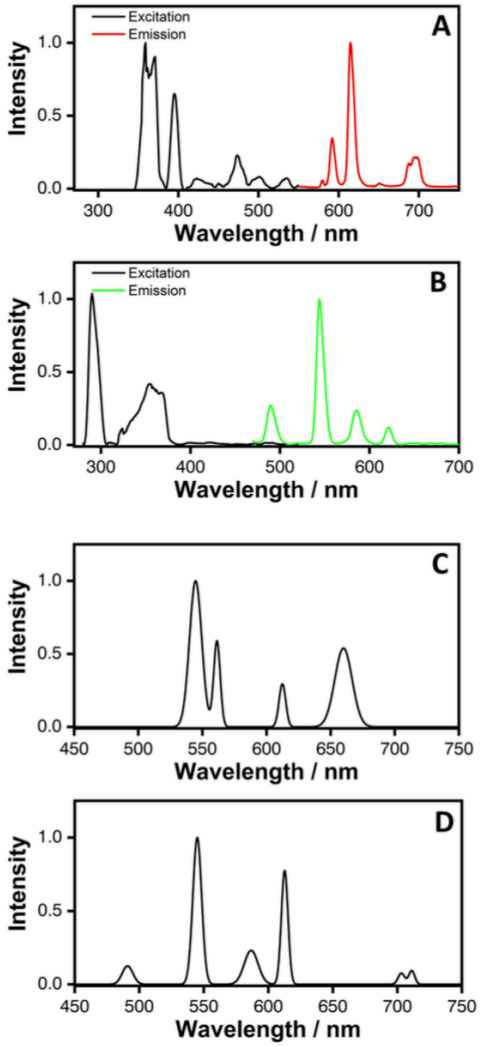

Figure 2. (A) Linear excitation and emission spectra of PBS®-614m. (B) Linear excitation and emission spectra of PBS®-544m. (C) IR upconversion emission spectrum of PBS®-UC660. (D) IR upconversion emission spectrum of PBS®-UC544.

The excitation and emission spectra of PBS® probes can be tuned during the proprietary biomineralization process. Figure 1A and B show the excitation and emission spectra of two colors of PBS® probes, PBS®-614 and PBS®-544, respectively. The emission spectrum of PBS®-614 shows an intense peak at 614 nm that has a full width at half max (FWHM) of 9 nm, along with several smaller peaks at 579, 592, 650, and 698 nm. The excitation spectrum for PBS®-614 shows intense peaks at 358 and 395 nm. The emission spectrum of PBS®-544 shows an intense peak at 544 nm with a FWHM of 10 nm and several smaller peaks at 490, 585, and 621 nm. The excitation spectrum of PBS®-544 shows a broad excitation band from 310 to 390 nm, with a sharper, more intense band at 290 nm. The narrow emission bands and the large shift between the excitation and emission bands of the two PBS® probe variants make it easy to isolate the signal from the PBS® molecules from other signals within the system, such as those resulting from autofluorescence, through the use of narrow bandpass filters. The narrow emission bands also make PBS® probes ideal for use in multi-colored experiments.

Through tuning the biomineralization process, two additional versions of PBS® probes named PBS®-UC660 and PBS®-UC544 have been developed. Unlike PBS®-544 and PBS®-614, which luminesce upon linear photoexcitation, PBS®-UC660 and PBS®-UC544 are infrared upconversion probes. The visible emission of both PBS®-UC660 and PBS®-UC544, shown in Figure 1C and D, respectively, is resultant from excitation at 980 nm. As with the linearly excited emissions, the upconversion emissions of PBS® probes exhibit sharp bands. There are several benefits to upconversion probes such as these. First, most tissues are opaque to visible and UV light, but are transparent to infrared light. This means that upconversion probes can be used to image these opaque samples. Additionally, autofluorescence and scattering from the excitation source can be issues with any linearly excited probe, but neither of these are issues with upconversion probes.

Luminescent Lifetimes and Time-Gated Techniques

PBS® probes are phosphorescent. Figure 2 shows the results of the luminescent lifetime measurements for PBS®-614 and PBS®-544. PBS®-614 has a 255 µs luminescence lifetime while PBS®-544 has a 1.5 ms lifetime. Most conventional probes, such as GFP and fluorescein, have fluorescence lifetimes between 1 and 20 ns (Table 1), which is in the same range as many of the endogenous fluorophores that contribute to autofluorescence. While it is possible to use FLIM to separate out the signals of the probe molecule and the autofluorescence, the similarities of the lifetimes can make such analysis difficult, particularly in instances when the signal from the probe molecule is weak. Because the phosphorescence lifetimes of the different variants of PBS® probes are orders of magnitude longer than the fluorescence lifetimes of the endogenous fluorophores, the signal from PBS® probes can be isolated using time-gated luminescence. In time-gated luminescence, a pulsed excitation source (either an LED or a laser) excites the sample. After the excitation pulse, there is a short delay time, termed the gate delay, prior to the measurement of the signal from the sample. The gate delay is chosen to be at least 10-times longer than the lifetime of any possible contributor to autofluorescence. Because the gate delay is so much longer than the lifetimes of the autofluorescence lifetimes, every molecule that contributes to autofluorescence will have returned to its ground state by the end of the gate delay.

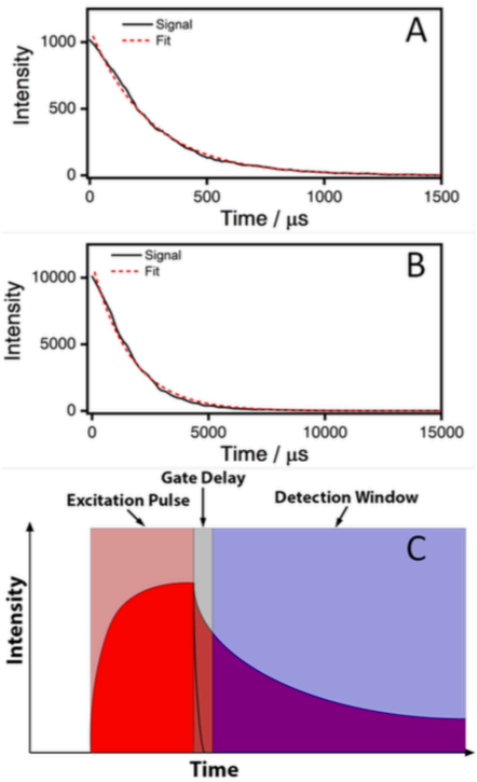

Figure 3. Luminescence lifetime measurements of (A) PBS®-614m (τ = 255 µs) and (B) PBS®-544m (τ = 1.5 ms). (C) Intensity versus time plot demonstrating how time-gated measurements work. A sample is excited with a pulsed excitation source. There is a delay between the excitation pulse (Gate Delay) and the detection window in which the fluorescence signal from all fluorophores with short lifetimes dies. Only signals from probes with long luminescence lifetimes are measured during the detection window.

This means that during the detection window, the only luminescent signals that will be measured are those coming from the phosphorescent probes. This dramatically increases the signal-to-noise ratios of measurements made using time-gated-luminescence when compared to measurements made using traditional fluorescence techniques. The increased signal-to-noise not only produces cleaner images, but it also makes it easier to detect weak signals, which is important when trace and ultra-trace detection is needed. When time-gated luminescence was applied to flow cytometry, it was demonstrated that rare-event of target cells were as effectively discriminated from the background as when the same sample was analyzed using multi-channel fluorescence flow cytometry (10-12). The time-gated flow cytometry measurement had the added benefit of only requiring a single label, compared to the multiple labels used for the multichannel fluorescence flow cytometry analysis.

Photostability and Shelf-Life

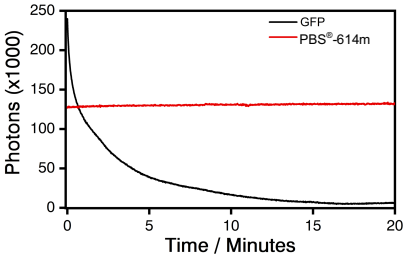

Figure 4. Comparing the photostabilities of PBS®-614m and GFP

The photostability of PBS®-614 was compared to that of GFP in solution. Both samples were excited at 395 nm for 20 minutes. The emission of GFP was monitored at 508 nm while the emission from PBS®-614 was monitored at 614 nm. Figure 3 shows a comparison of the time-dependent emission of GFP and PBS®-614. Initially, GFP was about twice as bright as PBS®-614. After about a minute, photobleaching had caused the intensity of the GFP signal to decrease by about 50%. By the end of the 20 minutes, the GFP sample was completely photobleached. The PBS®-614 sample, however, maintained a constant luminosity for the 20-minute period. Further experiments where PBS®-614 was continuously exposed to a 395 nm laser for 24 hours were performed, and the signal from PBS®-614 remained constant throughout the entirety of the experiment.

The ultrastability of PBS® probes gives them an unprecedented time-integrated level of detection. While it may have a lower luminosity (the number of photons emitted per second) than conventional probes like GFP or fluorescein, the total signal collected from PBS® probes can surpass that from other probes, as the other probes bleach over time.

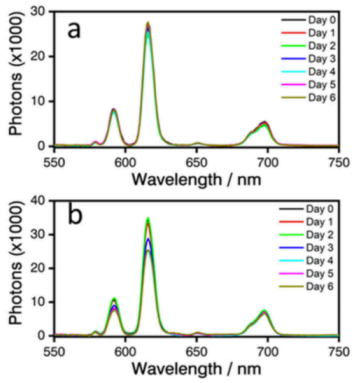

Figure 5. Spectra collected over a 1-week period showing the shelf-life of PBS®-614m stored at (a) 4 °C and (b) 25 °C.

PBS® probes are not only ultrastable, they also have a long shelf-life. The changes in intensity of the spectra of two PBS®-614 solutions stored at 4°C and 25°C were recorded over the span of a week. The results of these measurements are shown in Figure 4. The sample stored at 25° C only began to show a decrease in luminosity after Day 2, and by Day 6, the luminosity had only decreased by 26%. The sample stored at 4°C showed no significant decrease in luminosity over the period of time studied. The shelf-stability of PBS® probes was also studied using SDS-PAGE, which showed no degradation of PBS® probes stored at 4°C over a period of 3 months.

RECAL®'s Stability Offers a High Level of Time-Integrated Level of Detection

To demonstrate the sensitivity and high time-integrated level of detection that the photostability of PBS® technology offers, a PBS® probe was compared to a commonly used colorimetric probe in two different enzyme-linked immunosorbent assays (ELISA), one for aspartate aminotransferase (AST) and one for C-reactive protein (CRP). Both kits contained biotinylated detection antibodies and streptavidin-conjugated horseradish peroxidase (HRP) and 3,3',5,5'-Tetramethylbenzidine (TMB). For comparison, the streptavidin-conjugated HRP-TMB combination was replaced with avidin-conjugated PBS®-614. Figure 5 shows comparisons of the sensitivity of the ELISA tests using HRP-TMB and PBS® probes.

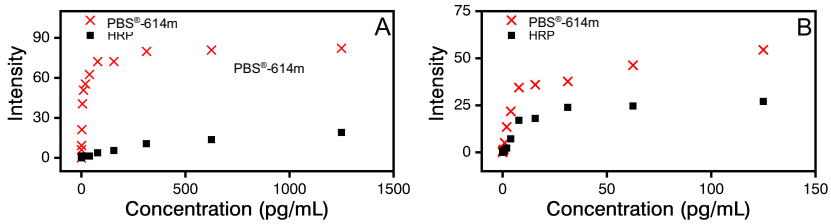

Figure 6. (A) Comparison of the sensitivity of the AST ELISA kits using HRP and PBS®. The ELISA kit using PBS® shows a 500-fold increase in sensitivity when compared to HRP. (B) Comparison of the sensitivity of the CRP ELISA kits using HRP and PBS®.

For AST, the PBS® probe-based assay showed a 500-fold increase in sensitivity over the HRP-TMB assay. For the CRP assay, the PBS® assay was 32-times more sensitive than the HRP-TMB assay.

Live Cell Imaging

Because PBS® probes are protein-based, it is also genetically expressible, with the biomineralization process taking place *in vivo*. PBS® probes were expressed in *E. coli* and a PBS® probe fusion protein (cytosolic) was expressed in HeLa cells.

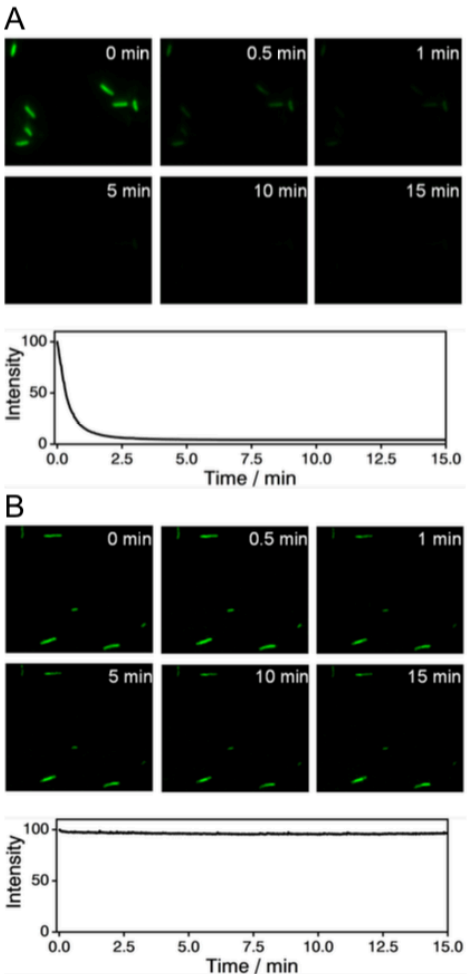

Figure 6. Fluorescent micrographs of *E. coli* expressing GFP (top) and *E. coli* expressing PBS®-614m under constant illumination.

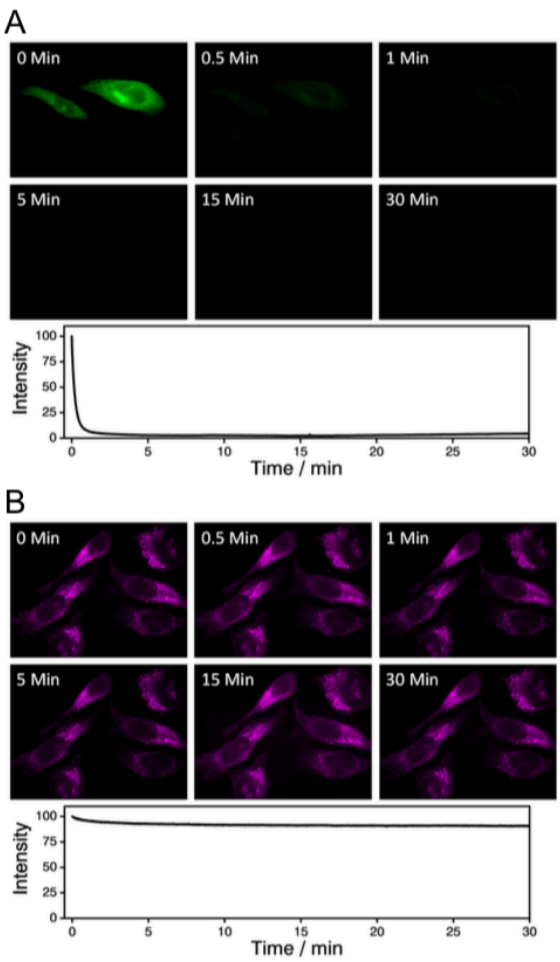

Figure 7. Fluorescent micrograph of HeLa cells expressing GFP-T05G5.9 (A) and HeLa cells expressing PBS®-614m-T05G5.9 under constant illumination.

Figure 6 shows time-lapse images of live *E. coli* expressing PBS®-614 and live *E. coli* expressing GFP. As was the case with GFP in solution, the GFP expressed in *E. coli* was bright, and the cells were easily imaged. After about 30 seconds of continuous light exposure, the brightness of the expressed GFP had greatly diminished, but the cells were still visible. After 1 minute of continuous exposure, some of the cells were no longer visible, and by 5 minutes, none of the cells expressing GFP were visible. In the *E. coli* expressing PBS®-614, the results were similar to what was observed in solution, as the signal stayed constant through the entirety of the experiment. It is worth noting that there is a small decrease in luminosity in the *E. coli* cells expressing PBS®-614 at the very beginning of the experiment. This is attributed to the bleaching of the *E. coli* endogenous fluorophores responsible for autofluorescence.

A mammalian expression vector for PBS® probes was constructed. In the mammalian expression vector, the PBS® probe-gene was fused to the gene for a large, soluble protein called T05G5.9. HeLa cells (ATCC®-CCL-2) were transfected with the gene for the fusion protein. For comparison, a GFP-T05G5.9 fusion vector was also constructed and transfected into HeLa cells. Figure 7 shows time-lapse images of the HeLa cells under constant illumination for 30 minutes. As with *E. coli*, the HeLa cells expressing GFP were initially bright and easy to image, but after only 30 seconds, the cells were significantly dimmer. After 1 minute, the cells were completely photobleached and impossible to image. Contrary to the HeLa cells expressing GFP, the cells expressing PBS®-614 were bright throughout the entirety of the 30-minute experiment. There is again a small decrease in intensity at the very beginning of the experiment. As with *E. coli*, this decrease in intensity is attributed to the bleaching of endogenous fluorophores present in the Hela cells.

The fact that PBS® probes do not bleach while being expressed *in vivo* opens many doors for researchers. For example, an individual living cell could be monitored for hours as they are exposed to various physical and chemical perturbations. This could be highly beneficial in the field of drug candidate screening, as it would allow researchers to monitor the long-time response of cancer cells to new drugs. A drug candidate screening experiment can be performed in much finer detail, allowing researchers to identify tipping points on *in vivo* drug screening effects.

Because PBS® probes are genetically expressible, the PBS® probe genes can be fused to the gene for a protein of interest for use as an expressible marker in much the same way that GFP, YFP, and RFP are, allowing PBS® to be used for the imaging of live cells undergoing apoptosis, necroptosis, pyroptosis, autophagy, and protein aggregation continuously. Additionally, anchorage independence (in 3D), cell motility and migration, marker expression, receptor internalization and degradation, sub-cellular localization, and translocation of transcription factors can be observed using PBS® probe gene fusions. The data collected from long-term live cell imaging with continuous light exposure can provide more predictive capability than the one-end point data usually associated with classic plate-reader HTS; therefore, continuous live cell imaging can be applied to many steps in the drug discovery workflow for the first time. The ability to image cells for longer time intervals by using our innovative, nonbleaching, luminescent biomarkers is advantageous for obtaining numerous data points from the same cells when screening large numbers of lead compounds to identify drug candidates.

Another drug screening technique where the PBS® probe's photophysical properties will be beneficial is live cell immunocytochemistry (ICC).(5) In this technique, antibodies against specific proteins in the cell membrane are labeled with a reporter marker, often a fluorescent molecule such as Atto 532, enabling visualization and tracking of membrane-bound proteins. This reporter-marker approach is used when the fusion of a fluorescent protein to a membrane-bound protein may inhibit incorporation of the membrane protein into the membrane (6). Such tracking, when combined with analysis of morphological and phenotypic parameters such as cell health, neurite outgrowth, and phagocytosis, allows for correlation of changes in expression of surface proteins with cellular function. Additionally, such methods make it possible to observe intercellular interactions and proximity relationships between motile cells. Incorporation of PBS® probes into these techniques would allow for the collection of an inordinate amount of data from numerous time points. Previously, such extensive data collection was not possible due to the photodegradation of the label molecules. Being able to collect so many data points for extended periods will help elucidate not only the observed interactions, but the mechanisms by which those interactions occur.

General Antibody Staining

While PBS® labelled antibodies can be used in above applications, it can also enhance general immunocytochemistry applications to obtain superior image quality with significantly reduced background. For example, HeLa cells were stained with an α-tubulin specific PBS® 544M conjugate and a nuclear protein H3 specific PBS® 614M conjugate (Figure 8).

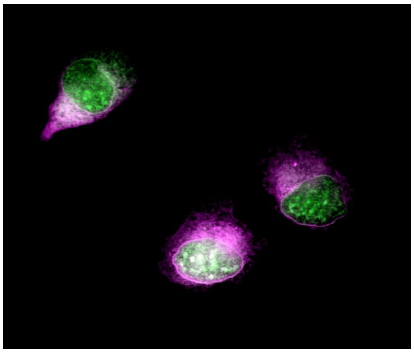

Figure 8. HeLa cells stained with α-tubulin and nuclear protein H3 specific PBS® 544M (magenta) and PBS® 614M (green)

HeLa cells (ATCC®-CCL-2) grown in DMEM in glass-bottom dishes were fixed and permeabilized by using an Image-iT™ Fixation/Permeabilization Kit (Invitrogen™). To the fixed cells, mouse anti-α-tubulin IgG (ThermoFisher Scientific, clone TU-01) and rabbit anti-H3 IgG (ThermoFisher Scientific, clone 24 HCLC) antibodies were added to a final concentration of 2 μg mL^{-1} and incubated for one hour. After incubation, the cells were washed three times with PBS supplied by the Image-iT™ kit and then incubated for one hour with a solution of PBS containing 2 μg mL^{-1} each of PBS®-614M conjugated to goat anti-mouse IgG and PBS®-544M conjugated to goat anti-rabbit IgG (secondaries purchased from Jackson ImmunoResearch). Finally, the cells were washed three times with PBS and imaged using an inverted Olympus IX-71 fluorescence microscope. The Xe/Hg arc lamp excitation source was passed through a 340-420nm shortpass filter (Semrock, FF01-424/SP-25) and focused onto the sample with an Olympus 40× oil objective. The luminescence from the sample was passed through a bandpass filter (Semrock, 615 nm bandpass, FF02-615/20; or Semrock, 544 nm bandpass, FF01-544/24), collected and imaged using a Hamamatsu ORCA II CCD camera.

Conclusions

We have demonstrated some of the ways in which PBS® probes are superior to many probes currently on the market. The excitation and emission wavelengths can be tuned during the biomineralization process. The emission bands of PBS® probes are narrow, allowing for the easy isolation of the signal from PBS® probes through the use of narrow bandpass filters. Because the luminescence of PBS® probes is phosphorescent, it is optimal for use in time-gated luminescence measurements, which can be used to minimize the background from autofluorescence. Additionally, the infrared upconversion variants of PBS® probes can be used to eliminate the problem of autofluorescence, and they can be used to perform measurements on specific samples, such as mammalian tissues and blood samples, that are opaque to much of the visible region of the electromagnetic spectrum. Unlike traditional probes, PBS® probes do not photoblink or photobleach, giving it an unprecedented time-integrated level of detection. This high time-integrated level of detection greatly increased the sensitivity of off-the-shelf ELISA kits when the detection reagent (HRP-TMB) was replaced by PBS® probes. PBS® probes are genetically expressible in both eukaryotic and prokaryotic cells and is non-toxic to cells. As was the case in solution, PBS® probes do not photobleach while expressed *in vivo*, allowing for the continuous imaging of cells, making it an valuable tool in live cell imaging related applications.

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