RECAL®: A New Ultrastable Luminescent Probe for Bioimaging

B.C. Borders[*], CH Kang[**] and J.A. Brozik[***]

[*]Photon Biosciences, LLC
445 S. Grand Avenue, Pullman, WA 99163, borders@photonbiosciences.com
[**]Washington State University, Pullman, WA, USA, chkang@wsu.edu
[***] Washington State University, Pullman, WA, USA, brozik@wsu.edu

ABSTRACT

RECAL® is a new, ultrastable, genetically expressible bionanoparticle that combines an engineered recombinant protein with a proporeitary biomineralization solution. Unlike traditional probes, RECAL® does not photoblink or photobleach. This gives RECAL® an unprecedented time-integrated level of detection. The excitation and emission bands of RECAL® are narrow and have a large Stokes shift, making it easy to isolate the signal from RECAL® using narrow bandpass filters. The excitation and emission bands of RECAL® can be tuned in the mineralization step and span much of the visible and near infra-red regions of the electromagnetic spectrum.

Here we show a comparison of the luminosity and stability of RECAL® and GFP. We also show a head to head comparision of RECAL® and GFP expressed in *E. coli.*

Keywords: microscopy, luminescence, protein, imaging, photobleaching, photoblinking

1 INTRODUCTION

Fluorescent proteins, quantum dots, and small fluorescent molecules are the three classes of commonly used fluorescent probes. All three types of probes can display large luminosity, but come with their own sets of advantages and disadvantages. While fluorescent proteins can be genetically expressed and fused to proteins of interest, they are not particularly stable and photoblink and photobleach [1,2]. The misfolding of these proteins during expression or during subsequent processing can also render them completely non-fluorescent. These proteins are also known to produce reactive O_2 radicals that can damage the cells expressing the proteins. Quantum dots are more photostable than fluorescent proteins, but still photoblink and undergo a process called bluing, whereby atoms are ejected from the nanoparticles while they are under illumination [3,4]. This is particularly problematic as most quantum dots contain heavy metals. Additionally, quantum dots are large and prone to aggregation, making their *in vivo* use problematic as they can disrupt cellular function [3]. While small fluorescent molecules are not genetically expressible, they are easily bound to biomolecules, proteins, and are small enough that they generally don't disrupt cellular function [3]. Fluorescent proteins also photoblink and are more susceptible to photobleaching than small organic probe molecules. In all three probes, autofluorescence, innate to most biological samples, decreases the signal-to-noise ratio of the measurements. While phosphorescent probes, through time delayed detection techniques, can alleviate the problem of autofluorescence, they are significantly less luminous.

We have developed a new ultrasensitive, ultrastable, genetically expressible bionanoparticle called RECAL®. RECAL® combines an engineered recombinant protein and a proprietary biomineralization solution. The excitation and emission wavelengths of RECAL® can be tuned by changing the biomineralization solution. Currently, there are two variantes of RECAL, one that emits primarily in the red, RECAL®-614m, and one that emits primarily in the green, RECAL®-544m. RECAL® is a phosphorescent probe, but its unique properties result in a luminescence intensity that approaches those of many fluorescent organic molecules. Here, we examine the spectroscopic properties of RECAL®, compare its photostability to that of green fluorescent protein (GFP) from *Aequorea victoria*, and demonstrate its use in *in vivo* imaging applications.

2 SPECTROSCOPIC PROPERTIES

2.1 Emission and Excitation Spectra

All laser-induced steady state spectra were measured using a tunable laser. The emitted light was dispersed by an Acton 500i monochromator and detected with a thermoelectrically cooled Hamamatsu R942-02 photomultiplier tube. For collecting the excitation spectra, light from a Hamamatsu Xe lamp was passed through an Acton 2300i monochromator.

Figure 1 shows the comparison of the excitation and emission spectra of RECAL®-614m, RECAL®-544m, and purified GFP. For emission spectra, RECAL®-614m was excited at 395 nm, RECAL®-544m was excited at 379 nm and GFP was excited at 420 nm. For the excitation spectra, RECAL®-614m was monitored at 614 nm, RECAL®-544m was monitored at 544 nm and GFP was monitored at 509 nm.

The emission spectrum of RECAL®-614m shows five peaks at 579, 592, 614, 650, and 698 nm, with the most intense peak at 614 nm. The peak at 614 nm has a full width half max (FWHM) of 9 nm. The excitation spectrum shows

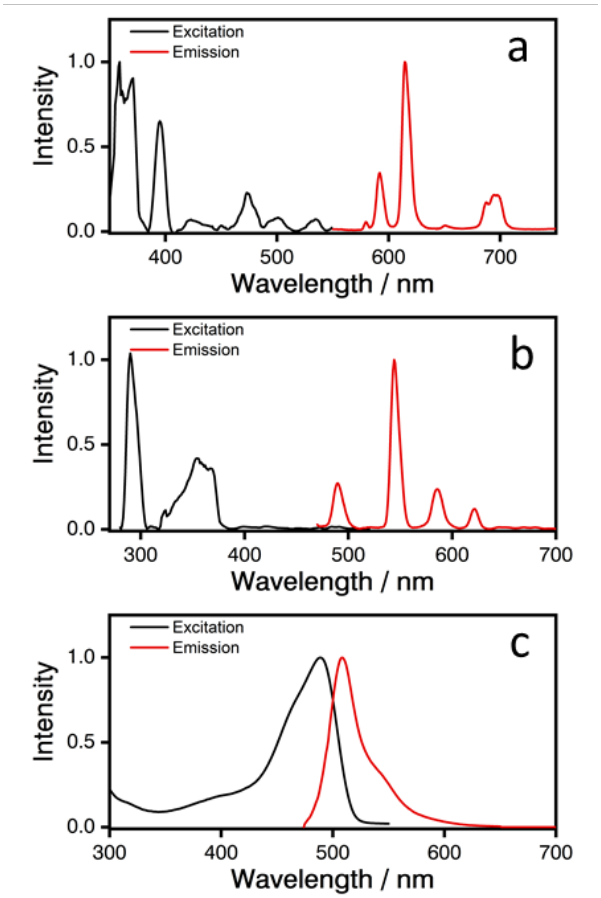

Figure 1. Excitation and emission spectra of (a) RECAL®-614m, (b) RECAL®-544m and (c) GFP

several bands, with the most intense peak at 358 nm, with another intense peak at 395 nm and a weak peak at 474 nm. The excitation peak at 395 nm has a FWHM of 10 nm.

The emission spectrum of RECAL®-544m shows four prominent peaks at 490, 544, 585, and 621 nm with the most intense peak at 544 nm. The peak at 544 nm has a FWHM of 10 nm. The excitation spectrum of RECAL®-544m shows three peaks: two overlapping peaks with maxima at 354 and 372 nm and an intense, narrow peak at 290 nm. The peak at 290 nm has a FWHM of 15 nm.

The emission spectrum of GFP shows a broad peak at 509 nm with a FWHM of 40 nm. The excitation spectrum of GFP shows a peak at 488 nm with a FWHM of 45 nm.

As expected, the excitation and emission bands of GFP are much broader than those of RECAL®-614m and RECAL®-544m. This broadness results in a loss of informational bandwidth. Additionally, GFP only has a Stokes shift of 20 nm, while RECAL®-614m and RECAL®-544m have a Stokes shift of 220 and 172 nm respectively. The large Stokes shift and narrow excitation and emission bands make it easy to isolate the signal from the two RECAL® variants from other signals in the system, such as those resulting from autofluorescence through the use of narrow bandpass filters. Additionally, the narrow bands greatly increase the informational bandwidth of RECAL®.

2.2 Luminescence Lifetime Measurements

The luminescence of RECAL® is phosphorescent in nature. The luminescence lifetimes were measured using the same setup described above for measuring the emission spectra, but with an optical chopper (Stanford Research, SR540) placed in front of the laser. The chopper was synchronized with the photon counter. Emissions were monitored at 614 nm and 544 nm for RECAL®-614m and RECAL®-544m respectively.

Figure 2 shows the results from the luminescence lifetime measurements. RECAL®-614m has a 255 µs lifetime while RECAL®-544m has a 1501 µs lifetime. Most conventional probes, such as GFP, have lifetimes in the ns range (see Table 1). The long luminescence lifetimes of RECAL® probes make them ideal for use in Fluorescence Lifetime Imaging, a technique frequently used to minimize the effects of autofluorescence. The main contributors to autofluorescence typically have fluorescent lifetimes between 1-20 ns [5]. The lifetimes of both RECAL®-614m and RECAL®-544m are long enough that they can be used in time-gated luminescence microscopy measurements, which can easily be performed by making simple modifications to conventional fluorescence microscopes. [6-7]

The long lifetimes of RECAL® probes also make them ideal for use in time-gated flow cytometry. The conventional approach for achieving highly selective descrimination of target cells in flow cytometry has focused on the use of multicolor biolabels and multifluorescent

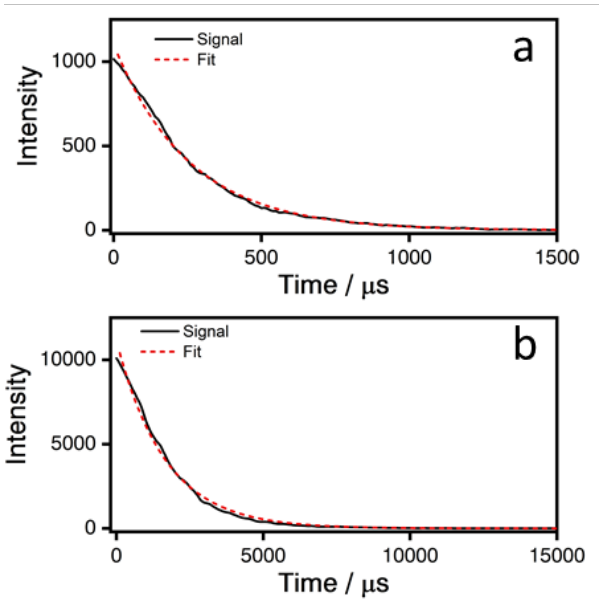

Figure 2. Luminescence lifetime measurements of (a) RECAL®-614m (τ = 255 µs) and (b) RECAL®-544m (τ = 1500 µs)

channel gating methods [8]. It has been demonstarted that rare-event target cells can be effectively discriminated from background by a time-gated method using long-lifetime luminsecent biolabels [9].

Fluorophore	Lifetime (ns)
GFP [10]	2.40
Atto 655 [12]	1.87
Alexa Fluor 532 [13]	2.53
Alexa Fluor 546 [13]	4.06
Cy 5 [12]	0.91
Rhodamine 6G [14]	4.0

Table 1. Fluorescence lifetimes of commonly used fluorophores

3 PHOTOSTABILITY AND SHELF-LIFE

The photostability of RECAL®-614m was compared to that of GFP. Both samples were excited with 2 mW 395 nm irradiation for 20 minutes. The emission from GFP was monitored at 508 nm while the emission from RECAL®-614m was monitored at 614 nm. Figure 3 shows the comparison of the emission of GFP and of RECAL®-614m over time. While GFP is certainly brighter than RECAL®-614m initially, within a minute the brightness of GFP has decreased to that of RECAL®-614m. At the end of the 20-minute period, the GFP sample was completely photobleached while the intensity of the RECAL®-614m was unchanged.

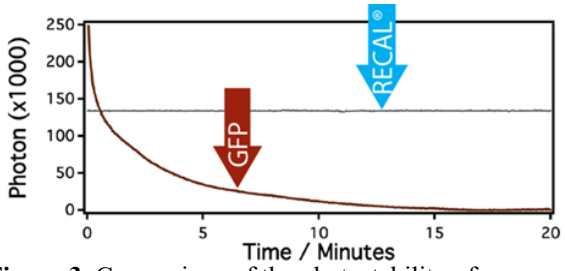

Figure 3. Comparison of the photostability of RECAL®-614m and GFP

The incredible photostability of RECAL® gives it an unprecedented time-integrated level of detection. The photostability, narrow emission profiles, long luminescent lifetimes, and luminosity combine to make RECAL® a truly unique probe with a signal that can easily be isolated from the background. This makes it possible to achieve detection at trace levels (20-50 pM) and even possible at ultratrace levels (20-50 fM). This will allow for not only conventional imaging/quantitative tools with increased precision, but also new studies, particularly in the field of single-molecule tracking and detection, that are not currently possible or are impractical with the technology that is currently on the market.

RECAL® also has an impressive shelf-life. The changes in intensity of the spectra of two RECAL® solutional

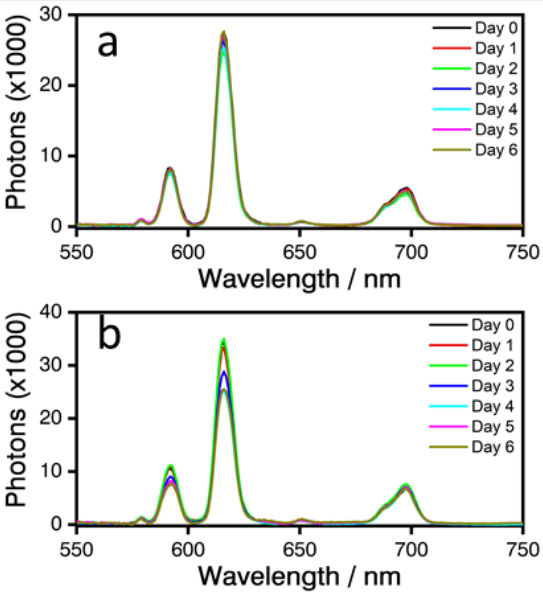

Figure 4. Spectra collected over a 1 week period demonstrating the shelf-life of RECAL®-614m stored at (a) 4° C and (b) 25° C

samples stored at 4° C and at 25° C were recorded over the span of a week. The results of these experiments are shown in Figure 4. The sample stored at 25° C only began to show a decrease in luminosity after 2 days, and by day 6 the luminosity had decreased by 26%. The the sample stored at 4° C did not show a significant decrease in luminosity over the period of time studied. The lyophilized RECAL displayed no significant reduction over six months (data not shown)

4 *IN VIVO* IMAGING

To demonstrate that RECAL® can be used for *in vivo* imaging, RECAL®-614m was expressed in *E. coli*. The imaging experiments were performed using a conventional fluorescence microscope. The excitation was set by a 420 nm (25 nm FWHM) bandpass filter and the luminescence was passed through a 600 nm (50 nm FWHM) bandpass filter and imaged onto a Hamamatsu ORCAII CCD camera. The LB broth was supplemented with the proprietary biomineralization solution. The measured growth curve did not show any sign of inhibitory effects as compared with a LB broth without the biomineralization solution. Likewise the mammalian cells did not show any inhibitory growth or shape (data not shown). Figure 5 shows a high resolution fluorescence micrograph of a drop of live *E. coli* expressing RECAL®-614m.

To demonstrate the superior photostability of RECAL® *in vivo*, samples of live *E. coli* expressing GFP were compared to samples of live *E. coli* expressing RECAL®-614m. The cells were held under constant illumination for 60 s. Figure 6 shows micrographs of the *E. coli* samples at the start of illumination, after 30 s of illumination, and after

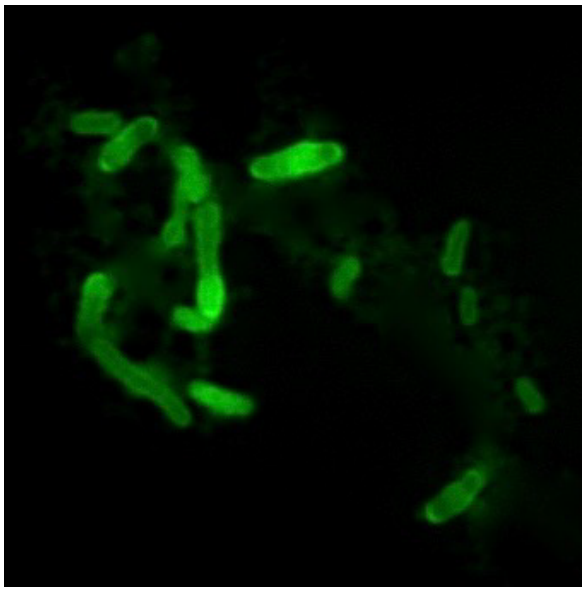

Figure 5. High-resolution luminescence micrograph of live *E. coli* expressing RECAL®-614m.

60 s of illumination. The *E. coli* expressing GFP started off bright, were dim after 30 s, and were almost completely dark after 60 s. The *E. coli* expressing RECAL-614 on the otherhand remained consistently bright throughout the imaging.

The high photostability of RECAL® is greatly beneficial to *in vivo* imaging. In addition to the increased sensitivity when using techniques such as FLIM, the fact that RECAL® doesn't photobleach means images can be acquired using longer exposure times without fear of bleaching the fluorophore. This will also allow for the low-level detection of proteins to which RECAL® is fused. Furthermore, RECAL® will allow for the imaging of a single cell for long periods of time, which will make it possible to perform studies that are currently impossible or impractical.

5 CONCLUSIONS

We have demonstrated the superior properties of RECAL®. The excitation and emission wavelengths can be tuned using the biomineralization kit and span much of the visible and near-infrared regions of the electromagnetic spectrum. Additionally, the excitation and emission bands are narrow, allowing for the use of narrow bandpass filters to easily isolate the signal from RECAL®. RECAL® is a phosphor with a long luminescence lifetime, making it ideal for time-gated measurements that can eliminate autofluorescence. Unlike conventional fluorescent probes, RECAL® doesn't photobleach or photoblink, giving it an unprecedented time-integrated level of detection. This combination of properties making it possible to achieve trace and even possibly ultratrace levels of detection using RECAL®. Additionally, the genetic expressibility of RECAL® make it possible for *in vivo* imaging experiments.

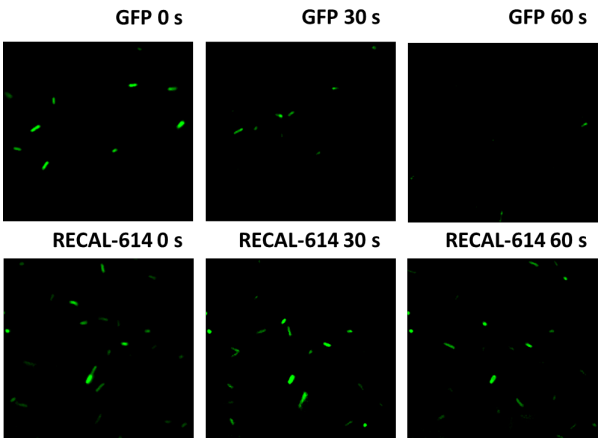

Figure 6. Fluorescent micrographs of *E. coli* expressing GFP (top) and *E. coli* expressing RECAL®-614m under constant illumination. Micrographs were collected at the start of illumination, after 30 s, and after 60 s

The incredible photostability of RECAL® make it possible to image samples using long exposure times and to monitor cells for long periods of time without fear of bleaching the probes.

REFERENCES

[1] E.S. Swenson, J.G. Price, T. Brazelton, and D.S. Krause, Stem Cells 25,2593, 2007
[2] E.C. Jensen, Anat. Rec., 295, 2031, 2012
[3] U. Resch-Genger, M. Grabolle, S. Cavaliere-Jaricot, R. Nitschk and T. Naan, Nature Methods, 5, 763, 2008
[4] S.F. Lee and M.A. Osborne, Chem. Phys. Chem., 10, 2174, 2009
[5] M.Y. Berezin and S. Achilefu, Chem. Rev., 110, 2641, 2010
[6] D. Jin and J.A. Piper, Anal. Chem., 83, 2294, 2011
[7] R.E. Connally, J.A. Piper, Anals. NY Acad. Sci., 1130, 106, 2008
[8] B.C. Ferrarai and D. Veal, Cytometry, Part A, 51A, 79, 2003
[9] D. Jin, J.A. Piper, R.C. Leif, S. Yang, B.C. Ferrari, J. Yuan, G. Wang, L.M. Vallarino, J.W. Williams. J. Biomed. Optics, 14, 024023, 2009
[10] A.A. Heikal, S.T. Hess, and W.W. Webb, Chem. Phys. 274, 37, 2001
[11] J.W. Borst, M.A. Hink, A. van Hoek, and A.J.W.G. Visser, J. Fluoresc. 15, 153, 2005
[12] V. Buschmann, K.D. Weston and M. Sauer, Bioconjugate Chem. 14, 195, 2003
[13] Y. Povrozin and E. Terpetscnig, Fluorescence Lifetime Measurements of BODIPY and Alexa Dyes on ChronosFD and K2; ISS, Inc.: Champaign, IL, 2009
[14] K.A. Selanger, J. Falnes and T.J. Sikkeland, J. Phys. Chem., 81, 1960, 1977