An analog method to produce time-gated images with lanthanide ion labels (P73)

Robert C. Leif, Sean Yang

*Newport Instruments, 5648 Toyon Road, San Diego, CA 92115
Phoenix Flow Systems, 6790 Top Gun St., Suite 1, San Diego, CA 92121

This poster is available for download at
http://www.newportinstruments.com

ABSTRACT

Problem: Lanthanide complexes have significantly lower extinction coefficients than organic fluorophores and require time-gated luminescence measurements to produce background-free images. Two solutions to the low extinction problem have been previously reported. 1) Resonance energy transfer enhanced luminescence (RETEL), particularly, in the solid state, allowed multiple antennae molecules to absorb light and then transfer the energy to a complexed lanthanide ion. 2) It has been possible to take advantage of the lack of concentration quenching of polymers of lanthanide complexes by the attachment of peptides containing multiple Lanthanide(III)-macrocycles to analyte-binding species A third solution is the use of recently developed lanthanide labeled nanoparticles.

Previous images of time-gated luminescence have been obtained with a cooled CCD camera by digitally summing a series of sequential images. The data acquisition rate of approximately 10 one millisecond exposure images per second was rate limiting and too slow for standard research and clinical use. These summed images contained an annoying undulating background, which could not be totally removed by subtraction of an unexposed, control image. Two inexpensive solutions to the analog summation of time-gated images have been developed. For both, a pulsed (ca. 500 Hz) inexpensive Nichia UV LED is used. The use of either an interline transfer, electronically shuttered camera or an inexpensive mechanical chopper permitted the analog summation of multiple images that were acquired during the dark period after each illumination.

Results and Conclusions: The interline transfer camera produced useful time-gated luminescent images of five micron and 0.5 micron uniform europium complex stained microspheres. The mechanical shutter system produced useful time-gated luminescent images of environmental pathogen microorganisms. The development of new chemistries together with improved instrumentation permit and encourage the use of lanthanide labels in cytometry. Either a software change to present commercial interline cameras can be made or a mechanical shutter can be employed to produce background-free, narrow spectral band width images of biological objects of interest. The use of lanthanide, background free labels is now both practical and desirable.

1. INTRODUCTION

- Lanthanide complexes: have very narrow emissions
- Have long lifetimes ca, 0.1 milliseconds to 2 milliseconds

  This permits the use of time-gated luminescence, which eliminates fluorescence background and contamination.

- Have large differences between their excitation (365 nm) and emission (619 nm) maxima (Stokes’ shift)

  Their emissions can be enhanced by resonance energy transfer enhanced luminescence (RETEL)\textsuperscript{1,2}

- Can be used at high local concentrations, because they do not concentration quench.

  Their emissions can be enhanced by being used in the form of highly labeled peptides\textsuperscript{3}.\n
Their emissions can also be enhanced by their inclusion in either organic or inorganic nanoparticles

- They have low molecular weights and sizes compared to quantum dots
- They do not blink

Table 1, Comparison of a Quantum Dot, a Quantum Dye (EuMac) and an Organic Dye (FITC-NCS)

<table>
<thead>
<tr>
<th>Label</th>
<th>Diameter Å</th>
<th>Volume Å³</th>
<th>Mass Atomic units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Qdot¹</td>
<td>116</td>
<td>825,800</td>
<td>1,500,000</td>
</tr>
<tr>
<td>EuMac²</td>
<td>15 Max</td>
<td>*504</td>
<td>674</td>
</tr>
<tr>
<td>Qdot-to-EuMac ratio</td>
<td>7.8</td>
<td>990</td>
<td>2,223</td>
</tr>
<tr>
<td>Small Phosphor Particles (3)</td>
<td>1,000-3,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-NCS³</td>
<td></td>
<td>389</td>
<td></td>
</tr>
</tbody>
</table>

¹Quantum dot. ²EuMac-mono-NCS. *Connolly solvent excluded volume from Chem 3D. ³FITC-NCS (Fluorescein isothiocyanate). ⁴“the luminescence intensity of the polystyrene nanoparticles to be about 10 times higher than that of Eu:Gd₂O₃ nanoparticles”⁵.

- Inorganic crystals have the problem that they do not contain a highly absorbing antenna species.
- However, they are more photostable than lanthanide complex containing plastic particles, and lanthanide complex labels.

The research and clinical use of lanthanide complexes as background free tags requires the acquisition of time-delayed images²,⁵.
Fig. 1. Emission spectra of fluorescein, the Eu Quantum Dye® (EuMac) and a 605 nm quantum dot. The spectra of the fluorescein and EuMac were obtained with a SLM spectrofluorometer with the excitation and emission slits set to 8 and 1 nm, respectively. The spectrum of the 605 nm quantum dot was redrawn from a spreadsheet obtained from www.qdots.com. The EuMac (1.0x10^{-5} M) was in an emulsion containing Gd(TTFA)$_3$; the fluorescein (1.0 x 10^{-5} M) was in hexamethylenetetramine buffer (1.0 M, pH 8.03). The spectra of both fluorescein and quantum dot were scaled to be of the same maximum height as that of the EuMac. The EuMac emission band-width is much narrower than that of either fluorescein or the quantum dot.
The acquisition period suitable for lanthanide complex stained cells is of the order of the half life of the luminescence. The half-life for the europium Quantum Dye® is 2 ms; and similar very long lifetimes have been observed by others. The lifetime of the FireRed™ microspheres is 0.46 ms.

Since an excitation period of one or two ms does not provide an adequate signal, it is necessary to sum multiple images.

Mechanical chopper, An image intensifier.

---

**Fig. 2** is a schematic diagram that shows a single cycle of a time-gated luminescence measurement. The lifetime of the lanthanide complex is assumed to be 250 microseconds. The excitation and decay are each shown for 1,000 μsec. The intensity of the emitted light is proportional to the number of excited lanthanide ions. The curvature of the intensity during excitations is the result of energy loss. Very quickly after the excitation UV LED is turned off, the short lifetime (< 0.1 μs) fluorescence ends. At this point the CCD is switched on and the image is acquired for 1,000 μsec. The luminescent decay has been assumed to be first order.
Digitally\textsuperscript{5}.

- Serial readout of the pixels and the digitization of the data permitted 10 frames per second. The actual acquisition time is 250 (2,500/10) seconds or 50 times slower.
- Each digital readout should have contributed 8 photo-electron equivalents or a total of 20,000 electrons equivalents.
- Summation of the background built-up readout noise & produced rollover of the 16 bit integers required change to 32 bit integers.
- Subtraction of an unexposed control image.
- This subtraction did not totally remove an artifactual horizontal undulation.

or Analog integration\textsuperscript{12} permits 1 millisecond for each excitation and signal capture period, 50\% duty cycle, 2,500 images = 5 seconds.

2. MATERIALS AND METHODS

2.1 Materials

**Nanoparticles:** Europium FireRed\textsuperscript{TM} 5 and 0.5 micron microspheres (www.NewportInstruments.Com) The DNA-check beads (www.beckmancoulter.com) PN: 6603488.

Equipment: Centrifugal cytology (Leif) buckets were equipped with 12 well inserts\textsuperscript{13}. 365 nm pulsed excitation from a Nichia 230mW LED (http://www.nichia.com/). Two monochrome progressive scan, interline, CCD cameras, each with a FireWire IEEE-1394 interface, were used for obtaining the images of the microspheres. One of these was a Dragonfly2 Point Grey Research (www.pointgreyresearch.com) with a 640 by 480 pixel, Sony 1/3” CCD Imaging Sensor (model ICX424AL) and Flycap software. The other was the previously used Retiga-1350 EX\textsuperscript{5} Peltier cooled, Quantitative Imaging Corporation (http://www.qimaging.com) 1280 by 1024 pixel, Sony 2/3” model ICX-285 CCD camera, which has a 12-bit ADC.

2.2. Methods

DNA-Check beads were centrifuged in 1.5 mL Eppendorf tube at 14,000 RPM for 10 seconds to pellet the beads. The beads were then resuspended with 0.5 mL dH\textsubscript{2}O. Thirty µL of the FireRed\textsuperscript{TM} 5 micron beads were added and the mixed suspension was sonicated for 10 second with Branson Ultrasonifier model 450 (http://www.sonifier.com) with micro-tip set at 10\% amplitude.

20, 40 and 60 µL of the bead suspension was sedimented in 12 well Leif Buckets on to a plain frosted end glass slide for 5 minutes at 300 x G. The supernatant was aspirated off and the 6 mm circular bead spots were air dried and coverslipped.

2.2.1. Time-gated luminescence microscopic imaging

The beads were imaged under UV (365 nm) LED excitation with a luminescence microscope\textsuperscript{5,14}. Images were obtained with essentially continuous squarewave excitation from a Nichia UV LED, model No. NCSU033A (http://www.nichia.com). The emission peak wavelength, half-width, and maximum optical power output were 365 nm, 10 nm, and 230 mW, respectively. A Laserlab power supply (http://www.laserlab.com) was used to drive the LED in pulsed mode and was controlled by a commercial signal generator. One millisecond-wide pulses were delivered at 500 Hz to power the LED. The emitted light traversed an Omega Optical (https://www.omegafilters.com/) PloemoPak cube UV DAPI, equipped with a 365-nm narrow bandwidth excitation filter Omega 365HT25 and a 400-nm beamsplitter Omega 400DCLP02. The optical path of the CCD was optionally equipped with a 619 nm narrow-band emission filter Omega 618.6NB.6.

2.3. Image Collection

**QImager Retiga -1350 EX Camera:** (http://www.qimaging.com) After each light pulse, all of the CCD image pixels were sequentially read-out, digitized, and the value of each pixel was digitally added to the sum of the previous read-
ings from that pixel (digital summation).

The Point Grey Research Dragonfly2 camera: (www.ptgrey.com) can operate in multiple modes. The Sony progressive scan CCD in the Dragonfly2 camera has two different types of pixels (http://www.datasheetcatalog.org/datasheet/sony/a6802973.pdf). The light sensitive pixels (square boxes) convert photons into electrons, which can be transferred to the storage pixels (vertical rectangles). The storage pixels are the ones that are part of the CCD transferring system that sequentially delivers the charge packets to the ADC. As shown in Fig. 3. Normally, after each time the

Fig. 3. Standard interline CCD. 1) The charge packets produced by the photons in each pixel (square boxes) are moved to the neighboring cell in the vertical register. Each vertical register reads out its bottom pixel to horizontal register and the horizontal register reads out to the ADC.
light sensitive pixels have been illuminated, their charge is transferred to the corresponding storage pixel. The storage pixels are then sequentially readout to the ADC. The two dimensional array is then transferred to the computer. This is essentially the way Point Grey Research’s Trigger Mode 0 (Fig. 4) operates and presumably the way the Retiga-1350 EX operates.

The essential difference for Trigger Mode 5 is the sequential delivery of the charge packets to the ADC does not occur after each image has been acquired; it only occurs after the last image has been acquired. Mode 5 permits (Fig. 5) the analog summation of the pixels from each line and the use of an electronic shutter, which limits the acquisition of light to the period when the light is off. Thus, the acquired image only includes the noise from one readout Trigger Mode 0.

Fig. 4. Chart describing a single image taken in Trigger Mode 0. A) The UV light is controlled by the square wave produced by the signal generator. The CCD camera’s electronic shutter (B) is on for the period of the exposure, 66 milliseconds. The square wave of UV light is on continuously. In principle, the UV light could have been unmodulated. C) The light sensing pixels during each light pulse increase their charges. The break after 8 milliseconds is to permit showing the full duration of the exposure. After the exposure, the charges are directly readout through the two dimensional array of storage pixels, and digitized to form the image (D). Since the duty cycle was 50%, the actual exposure was for 33 milliseconds.
2.3.1 Trigger mode 5

Fig. 5. Chart describing Mode 5. A) The UV light is controlled by the square wave produced by the signal generator. The pulse width was constant for all of the images that were summed. B) State of the CCD light sensor pixels. When the polarity of the square wave is low the CCD camera is on. The first external pulse falling edge (A) turns the electronic shutter of the light sensitive pixels (B) on, which permits the incoming luminescent emission to be converted to electrons in the photosensitive pixels. When the polarity starts to switch to high, the contents of the row of sensing pixels are, as shown in (C) transferred to the corresponding row of light insensitive pixels in the storage array, and the electronic shutter stops the signal from being accumulated by the sensing pixels. The break after the eighth pulse indicates that multiple pulses have occurred between the two sections of the pulse train. Only after the last pulse (100 milliseconds in this figure) is the summation of the pulses in the storage pixels sequentially read out and digitized to form the image (D). In the figure, the number of images (pulses) is shown as being set for 50.
3. RESULTS

3.1 Retiga Camera Studies

![Fig. 6. Digitally summed background (emission blocked) images obtained with the Retiga camera. The number of one millisecond exposures summed are shown at the top upper left. For all of these images, the number of readouts equals the number of exposures. All of the multi-readout images except for the 1,000 exposures images were cropped to the area shown in the upper left of the 1,000 exposure-readouts image. These background images show a periodic undulating horizontal pattern, whose contrast increases with the number of images. The 1000 x1 image shown at the lower right consisted of 1,000 light pulse exposures, but only 1 readout. It shows a few white defective pixels but does not show an evidence of the periodic horizontal pattern.](image)

The periodic horizontal pattern shown in Fig. 6 is the result of the readout process. As is shown, it becomes visually more distinct as the number of readouts increases and does not exist in the control image that was taken for 100 seconds, which is the amount of time it takes the Retiga to accumulate and sum 1000 (1000x1) exposures.

3.2 Dragonfly2 Study, Five micron microspheres

![Fig. 7. Comparison of conventional and analog summed inverted time-gated images. All images were obtained with the 40x N.A. 0.65 objective and a Nichia UV LED which produced 500 pulses per second, each of which with a duration of 1 ms. each for excitation and subsequent image acquisition. The Eu(TFFA)$_3$ stained 5 micron (smaller) microspheres and the larger microspheres are DNA-check microspheres. The left and center images had respectively 619 nm and 545 nm emission filters and were obtained as single images in Mode 0. No emission filter was employed for the summation of 50 images (right), which were obtained in Mode 5. The contamination of the emissions of the larger DNA-check microspheres in the image obtained with the 619 nm emission filter is comparable to that in the image consisting of 50 analog summed](image)
images. The emission of the europium microspheres recorded in the analog summed image is greater than that obtained with the 619 nm emission filter. Thus, a useful time-delayed luminescence image could be obtained with a total excitation and signal capture time of 0.1 seconds, which is comparable to a single readout of the camera.

### 3.3 Half (0.5) micron microspheres

As is shown in Fig. 8, because of the weakness of the emissions from the individual microspheres, the number of images that need to be summed had to be increased in order to create a printable image resulted in significant background noise.

The locations of and numbers of microspheres shown in the outlined drawings 50P and 100P (not shown) are virtually identical and consistent with the locations of the microspheres.

![Images showing the effect of summing microsphere images](image)

**Fig. 8.** Centrifugal cytology dispersions of 0.5 micron FireRed™ microspheres. All images were obtained with the 60x, N.A. 1.25, oil immersion objective and a Nichia UV LED which produced 500 pulses per second (1 ms for excitation followed by 1 ms for image acquisition). The upper left image had a 619 nm emission filter and was obtained as a single image in Mode 0. No emission filter was employed for the other summed images, which were all obtained in Mode 5. The number in the upper left-hand corners of the other images is the number of individual images that were analog summed. The 50P is the ImageJ processed image of image 50. A similar image (not shown) with the identical outlines was made for the 100 pulses image. These processed images were first thresholded and then analyzed.

As is shown in Fig. 8, because of the weakness of the emissions from the individual microspheres, the number of images that need to be summed had to be increased in order to create a printable image resulted in significant background noise.

The locations of and numbers of microspheres shown in the outlined drawings 50P and 100P (not shown) are virtually identical and consistent with the locations of the microspheres.

![Images showing background noise](image)

**Fig. 9.** Background images obtained with the excitation light path blocked. The number in the upper left-hand corner is the number of images that have been analog summed.

**Fig. 9 shows the background noise images from the upper left-hand corner of the controls for Fig. 8. The noise becomes significant at about 200 images (equivalent of an exposure of 0.2 to 0.4 seconds). The relative noise contribu-**
tion of the image acquisition and storage pixels is unknown. Both would be greatly diminished in a comparable scientific cooled camera.

4. CONCLUSIONS

- The analog integration solution apparently works, should be reliable and inexpensive; however, the rate of noise generation is sufficiently high that the present camera is unsuited for the detection of many samples of interest, which are weakly labeled.
- A cooled scientific grade camera with the same capacity for analog summation should be used.
- The only additional costs are for a software modification to a commercially available camera and the circuit used to pulse the UV LED.
  - insignificant compared to the cost of a present fluorescence microscope camera combination.
  - The narrow emission and time-gating could be combined with a lower noise research grade, cooled camera
- A very inexpensive system could be constructed employing a simple bottom to top illumination system because the large Stokes' shift of europium complexes together with time-gating should minimize the noise produced by the excitation light.
- The use of a special camera described in this paper has the two disadvantages compared to the controlled aperture (chopper) approach of Jin et al.\textsuperscript{9,16}
  - For their approach\textsuperscript{9,16}, a commercially available unmodified camera can be used.
  - It also permits observation of the cells through the eyepieces.
- Either low cost technical solution to time-gated luminescent imaging can be combined with multiple forms of lanthanide luminescent labels. These include organic and inorganic lanthanide containing nanoparticles, densely labeled polymers\textsuperscript{3}, conventional functionalized single metal-organic complexes, and Resonance Energy Transfer Enhanced Luminescence (RETEL)\textsuperscript{5}.
- In short, the use of lanthanide, background free labels is now both practical and desirable.

5. REFERENCES


