Time-Gated Luminescence Microscopy

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Autofluorescent algal samples were spiked with europium beads for analysis on a novel all-solid-state, time-gated luminescence (TGL) microscope. Pulsed UV excitation (365 nm) was provided by a high-power UV-LED source fitted to an Olympus BX51 microscope. An "Impactron" electron multiplying charge-coupled-device (CCD) camera acquired images in delayed luminescence mode. Second, we evaluated sensitivity of the instrument by acquiring images of immunofluorescently labeled *Giardia* cysts with a single-exposure period of 3 ms. The camera was triggered 3 μs after the LED had extinguished to yield a 14-fold increase in signal-to-noise ratio within a single 33 ms capture cycle. This novel instrument could be switched instantly from prompt epifluorescence mode to TGL mode for suppression of short-lived fluorescence.

Key words: **time-gated luminescence; autofluorescence; lanthanide; phosphorescence; *Giardia*; EMCCD; UV LED**

Introduction

Fluorescence-based techniques provide a powerful means for both the qualitative and quantitative detection of biomolecules.[2] Fluorescence methods afford a sensitive means for the detection of single molecules,[5] however probes lose much of their discriminatory power in the presence of autofluorescence. Organic and inorganic autofluorophores are ubiquitous in nature and some materials fluoresce with great intensity, obscuring or diminishing the visibility of synthetic fluorescent probes.[7] Spectral selection techniques (emission and excitation filters) can reduce the problem but are not always applicable due to the abundance and spectral range of autofluorescence.[9]

Time-gated luminescence (TGL) techniques operate within the time-domain and are directed toward detection of events that occur at much longer timescales (phosphorescence).

For TGL operation, the detector is gated off while a brief pulse of light is used to excite emission from the sample. The detector is maintained in the off-state for a resolving period (gate-delay) while short-lived (<1 μs) fluorescence fades beyond detection. The detector is then enabled to capture luminescence in the absence of autofluorescence, greatly increasing the signal-to-noise ratio (SNR) (FIG. 1).

While it is possible to discriminate probe fluorescence from autofluorescence using time-resolved fluorescence (TRF) techniques, a simpler and much less costly TGL instrument can be employed if a suitable luminescent probe is available. Lanthanide (Eu^{3+} or Tb^{3+}) chelate luminescent probes have exceptionally long phosphorescence lifetimes (τ) reaching milliseconds for some compounds.[12] Other compounds that have found wide application for TGL include the platinum and palladium (copro)porphyrins, with lifetimes ranging from 10 to 1000 μs depending on their environment. The very large difference in τ between typical autofluorophores and the luminophores used for TGL has helped ensure useful results were gained even with simple instruments relying upon chopper-wheels.

The substantial increase in SNR afforded by TGL techniques is a critical factor when searching for rare target organisms encountered in autofluorescent environments. For example, the detection of *Cryptosporidium* oocysts in drinking water requires the filtration of large volumes of water and results in a matrix of mineral particles, algae, desmids, and plant matter that is strongly autofluorescent. TGL microscopy has been demonstrated to greatly suppress this background and simplify the detection of both *Giardia* and *Cryptosporidium*, two important waterborne pathogens. There are instances where the detection of rare-event signals using conventional fluorescence techniques is exceedingly difficult (or impossible), and consequently, where TGL microscopy has greatest utility.[17–20]

Luminescent probes based on the lanthanides Eu^{3+} and Tb^{3+} were described in the 1960s, but effective immunofluorophores using these compounds were not

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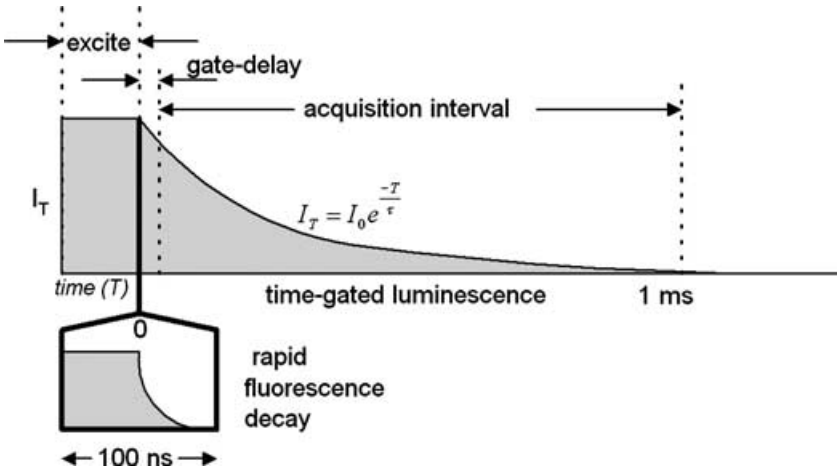

FIGURE 1. Pulse fluorometry or time-gated luminescence techniques operate on timescales orders of magnitude greater than frequency domain methods. Short-lived fluorescence ceases within nanoseconds after excitation whereas phosphorescent emission can persist for several hundred microseconds.

reported until the early 1980s. TGL microscopes were built to exploit these novel compounds; however various deficiencies in the instrumentation and luminescent probes resulted in relatively insensitive instruments. As technologies matured, improvements were made both in instrument design and probe quality. The evolution of TGL microscope instrumentation is discussed briefly here to provide some insight into the shortcomings of previous incarnations and to illustrate the utility of our new instrument. In the following discussion, we have reviewed only TGL microscopes that are designed for the detection of phosphorescence ($\tau > 10\ \mu s$).

Evolution of TGL Microscope Instrumentation

TABLE 1 concisely summarizes the evolution of TGL instrumentation over the last 20 years, beginning with Soini's pre-TGL instrument, which only hinted at the true power of this technique, and concluding with the entry of the first all solid state TGL microscope.

With reference to FIGURE 1 it can be seen that TGL techniques employ an excitation pulse to excite photon emission from the sample. This pulse ideally terminates abruptly ($<1\ \mu s$) while the detector is maintained in the off-state for a resolving period, the duration of which is designed to permit short-lived fluorescence to decay. After the resolving period (gate-delay) has elapsed, the detector is gated on (acquisition period) to capture signal from the luminescent label, free of background noise.

TGL Excitation

The choice of excitation source wavelength is largely dependent on the target luminophore; most lanthanide chelates require UV excitation in the range 300 to 370 nm. Platinum and Palladium chelates can be excited around 400 nm, Ruthenium at 475 nm, Iridium at 310 nm, Chromium at 420 nm, and Osmium at 480 nm.[25,26] The transition metals (Ru, Ir, Cr, and Os) are not commonly employed for TGL work due to their relatively short luminescence lifetimes ($<1\ \mu s$). Europium (Eu^{3+}) luminescence is centered around 618 nm ($\tau \cong 600\ \mu s$); Terbium (Tb^{3+}) has a major peak at 545 nm and two smaller peaks at 495 nm and 590 nm ($\tau \cong 800\ \mu s$), see Bunzli *et al*. for a recent review.[12] Platinum coproporphyrins emit at 647 nm ($\tau \cong 80\ \mu s$), and palladium at 667 nm ($\tau \cong 700\ \mu s$).

Chopper Wheels

In 1988 Soini and colleagues described a novel europium chelate (W1014) that could be easily bound to biomolecules to permit them to "re-test the old idea of time-resolved fluorescence microscopy in immunohistology and cytology." Using steady-state excitation, it was shown that Eu-antibody-labeled histology sections were visibly luminescent to the naked eye and would likely provide a means to improve SNR under TGL conditions.[1] The following year, Beverloo and others[3] described a Xenon flashlamp-excited TGL microscope synchronized to a chopper wheel. As a model for this work, highly luminescent immunoconjugates were prepared by adsorbing various antibodies to the surface of Y_2O_2S:Eu phosphor particles. Luminescently labeled regions were readily apparent to the naked eye, and autofluorescence suppressed 100-fold

TABLE 1. History of the development of time-gated luminescence (phosphorescence) microscopes

Date	Author	Excitation	Detection	Luminophore
1988	Soini *et al.*[1]	Hg lamp, CW	Eye	Eu^{3+} chelate (W1014)
1990	Beverloo *et al.*[3]	Xenon flashlamp	CCD + chopper	Y_2O_2S:Eu
1992	Seveus *et al.*[4]	Xenon flashlamp	CCD + chopper	Eu^{3+} chelate (W1014)
1994	Marriot *et al.*[6]	Hg lamp + chopper	CCD + chopper	Eu^{3+} BCPDA
1994	Verwoerd *et al.*[8]	Xe-arc + chopper	CCD+ferro-electric shutter	Porphine labeled Sephadex
1994	Seveus *et al.*[10]	Xenon flashlamp	CCD + chopper	W-8044 Eu^{3+} chelate
1995	Phimphivong *et al.*[11]	He-Cd laser + chopper (400 Hz)	CCD + ferro-electric shutter	Tb^{3+} chelate
1996	Hennink *et al.*[13]	Argon-ion laser + AOM	Gated MCP image intensifier + CCD	Pt-porphine
1996	de Haas *et al.*[14]	Xenon flashlamp	CCD+ferro-electric shutter	W-8044 Eu^{3+} chelate
1997	Rulli[15]	Xenon flashlamp	Gated CCD	Eu^{3+} conjugated to anti-human IgG
1997	de Haas *et al.*[16]	Argon-ion laser + AOM	Gated MCP image intensifier + CCD	Pt porphyrin excited at 514 nm
1998	Vereb *et al.*[21]	Xenon flashlamp	Gated MCP image intensifier + CCD	Eu-DTPA-CS124
1998	Phimphivong *et al.*[22]	He-Cd laser + chopper (400 Hz)	CCD+ferro-electric shutter	Tb^{3+} p-amino salicylaldehyde
1999	de Haas *et al.*[23]	Hg-arc + chopper	CCD+ferro-electric shutter	Pt and Pd co-proporphyrins
2002	Connally *et al.*[9]	Xenon flashlamp	Gated MCP image intensifier + CCD	Eu^{3+} - BHHCT
2003	Soini *et al.*[24]	Xenon flashlamp	CCD + chopper	Eu^{3+}, Tb^{3+}, Pd and Pt coproporphyrins
2004	Connally *et al.*[27]	Xenon flashlamp	Gated MCP image intensifier + CCD	Eu^{3+} - BHHST
2006	Connally *et al.*[28]	UV LED	Gated MCP image intensifier + CCD	Eu^{3+} - BHHST
2007	Connally *et al.* (in press)	UV LED	EMCCD	Eu^{3+} - BHHST

over standard epifluorescence microscopy. Limitations of the system were attributed to the relatively large size of the phosphor particles that proved problematic in the preparation of immunoconjugates.[3]

In collaboration with Soini in 1992, Seveus and colleagues[4] described construction of a Xenon flashlamp-excited TGL microscope. The W1014 europium chelate described 4 years earlier was used to label cancerous cells. Unfortunately, this new chelate was found to present stability problems under excitation and in connection with different reagents used for counterstaining.

Luminous intensity of the label was reduced compared to that achieved with Beverloo *et al*.'s phosphor nanoparticles, and detection was by means of a sensitive cooled charge-coupled-device (CCD) sensor fitted with a chopper wheel synchronized to the flashlamp.[4]

Two Wheels Better than One?

A TGL microscope employing two phase-locked chopper-wheels for pulse control (detection and excitation) was reported by Marriott and co-workers in 1994.[6] FIGURE 2 details the essential features of such an instrument with the chopper-blades positioned in their respective states for the excitation and emission phases of a TGL cycle. A 100 W mercury arc lamp was interrupted by the chopper blades to deliver the pulsed UV excitation required by the europium chelate BCPDA. The CCD detection-side chopper was phase locked to the excitation-chopper unit by a control module that could maintain an arbitrary phase difference between the two. The instrument could be switched from delayed luminescence mode to "prompt" mode by adjusting this phase angle. The shortest gate-delay that could be achieved with this instrument was 210 μs, a serious limitation given that BPCDA has a luminescence lifetime ranging from 210−745 μs (depending on environment).[6]

Chopper Wheels and the Effect on Gate-Delay

The gate-delay is the intervening period between termination of the excitation pulse and commencement of the acquisition phase (see FIG. 1). For most phosphorescent labels, decay follows single exponential kinetics ($I_0 = I_t e^{-t/\tau}$), and emission decays substantially as the gate-delay interval approaches the luminescence lifetime. For example, the BPCDA chelate used by Marriott *et al*.[6] with τ of 210 μs in phosphate-buffered saline solution would suffer a loss of 63% of the

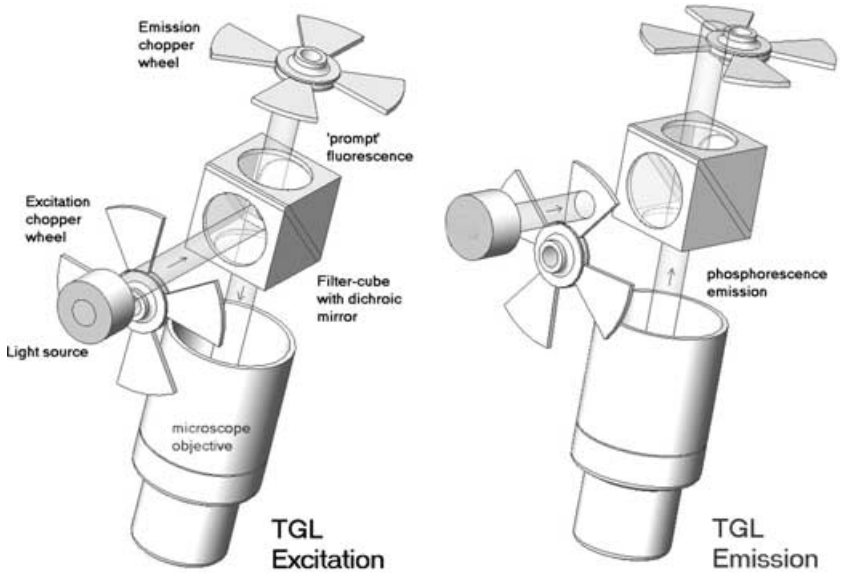

FIGURE 2. The long persistence of lanthanide phosphors makes feasible the use of mechanical choppers for visualizing the phenomenon. A number of early TGL instruments employed chopper wheels to isolate the excitation and detection phases in a TGL cycle. The figure on the left shows the chopper blade arrangement during excitation mode, light source open, and detector closed. The right-side figure depicts the path of subsequent luminescent emission as it propagates toward the exposed detector.

initial intensity if acquisition began after a 210 μs gate-delay. Unfortunately, gate-delays of this magnitude are typical for chopper-based switching mechanisms.

Electronic Shutters

A good example of the adoption of new technologies for TGL microscopy was given by Verwoerd and colleagues in 1994.[8] Ferro-electric liquid crystals (FELCs) rotate the plane of light polarization in response to an applied voltage and can serve as fast optical shutters. A TGL microscope was constructed in which the emission-plane chopper was replaced with two crossed LC shutters. For excitation, a Xenon-arc lamp was interrupted by a mechanical chopper to generate pulsed output; gating of the LC shutters was synchronized to the chopper wheel position. Fluorescein-labeled beads were used to model an autofluorescent background from which phosphorescent, porphine-labeled Sephadex beads were resolved in high contrast. While effective, the FELC shutters imposed a substantial insertion loss, with transmission reduced to just 15% when fully open. A further limitation of chopper excitation schemes arises from the relatively slow rise and fall time of the pulse, which in this case was 50 to 100 μs at a disk speed of 3800 rpm. Excitation pulses with slow falling edges force extension of the gate-delay that leads, ultimately, to a loss in SNR. The gain achieved by switching rapidly in the emission plane was offset to some extent by the slow falling edge of the excitation pulse. Nevertheless, the authors described a similar design 5 years later in which they retained the excitation chopper but relocated the FELC shutter to the filter holder to permit observation of delayed luminescence with the naked eye.[23]

Flashlamp

Luminescence signals observed with TGL microscopes are comparatively weak compared to the output from a continuously excited fluorophore observed in a conventional fluorescence microscope. Flashlamps conveniently deliver substantially more energy in the excitation pulse within a shorter time interval (2−10 μs) compared to a chopper-interrupted continuous wave (CW) source. As a result, flashlamp-equipped TGL microscopes excite stronger emission and deliver superior SNRs. In addition, flashlamps are relatively inexpensive and vibration free, factors that have led to their incorporation into a number of TGL microscope designs,[21,27] including a recent instrument manufactured by Perkin Elmer.[24]

Minor deficiencies of flashlamps include a limited repetition rate, variability in pulse-to-pulse energy, and a limited lamp lifetime. The major drawback to their use relates to the plasma glow that persists following the primary arc-discharge. This glow can persist for many tens of microseconds, decaying exponentially and forcing the gate-delay to be extended for 50 μs or more.[27] The extended gate-delay necessary with

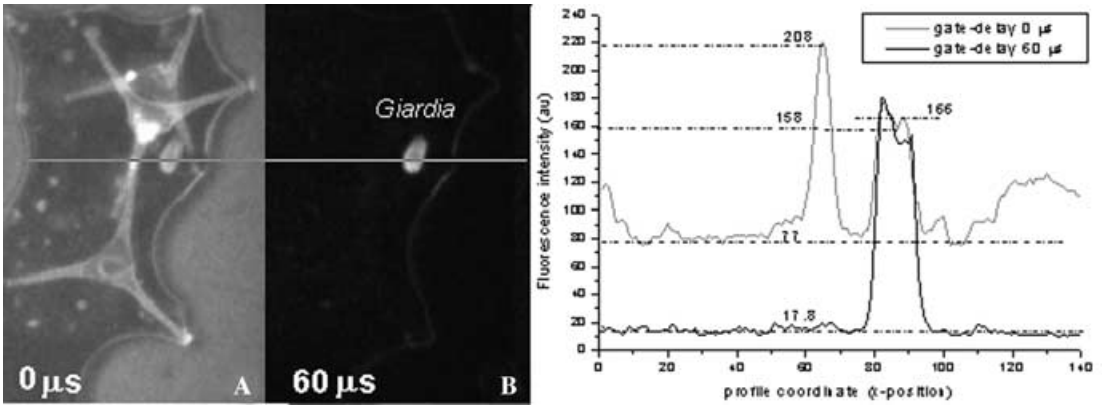

FIGURE 3. Flashlamps can deliver an intense burst of light and are superior pulsed sources compared to chopper-wheel/lamp combinations. An immunofluorescently labeled *Giardia* cyst is shown surrounded by autofluorescent desmids in conventional epifluorescence mode. Autofluorescence is strongly suppressed in the image on the right, which was acquired with a flashlamp-excited TGL microscope. Plasma glow that persists from the flashlamp following discharge required the gate delay be extended to 60 μs. The line transecting the image indicates the path of the line profile shown on the right-hand panel. Autofluorescent regions mapped in conventional epifluorescence mode (light grey) are strongly suppressed in TGL mode (dark grey) to significantly improve the SNR.

flashlamp-excited instruments is not a critical limitation when long-lived, high-yielding luminophores are employed. FIGURE 3 shows an image of a *Giardia* cyst labeled with the europium chelate BHHST, acquired with a flashlamp-excited TGL microscope using a gate delay of 60 μs. BHHST has $\tau \cong 300\,\mu s$, and a gate-delay of 60 μs imposes a 20% loss on label intensity before detection commences. While autofluorescence from the desmids was suppressed 11-fold, the SNR could have been enhanced were it possible to implement a shorter gate-delay interval.[9]

UV Laser

To achieve maximum luminescence intensity, it is necessary to ensure that the majority of luminophores are raised to an excited state (a population inversion) following the excitation pulse. The intense radiance of a laser beam can help ensure that a population inversion is achieved within the excitation interval. Lanthanides typically require excitation in the UV (~340 nm) and respond to the 337 nm output from an N_2 gas laser. The N_2 laser has found application in TRF microscopy[29,30] and TGL fluorometry,[31] but a TGL microscope using this excitation source has not been reported. He–Cd gas lasers are relatively inexpensive and emit UV at 325 nm, suitable for excitation of Tb^{3+} chelates. A TGL microscope utilizing chopper-interrupted output at 400 Hz from a He–Cd laser source was described by Phimphivong and colleagues in 1995.[11] The instrument was especially adapted for total internal reflection TGL microscopy and used a similar LC shutter arrangement to that described by Verwoerd *et al*.[8] It was necessary to collect the relatively weak luminous emission from the Tb^{3+} label over hundreds of TGL cycles on a cooled CCD detector.[11]

Visible Laser Excitation

In a refreshingly new approach, Hennink and coworkers employed the output from an acousto-optical modulated (AOM) argon-ion laser as the pulsed excitation source for a TGL microscope described in 1996.[13] Platinum and palladium porphyrins possess a strong absorption peak at 390 nm and a second weaker peak at 530 nm. Argon-ion lasers are relatively low-cost sources with visible output at 514 nm suitable for exciting this secondary absorption peak. An advantage of using visible wavelengths is the possibility of coupling the laser AOM output directly to the microscope via an optical fiber. A further innovation pioneered by Hennink's team was the use of a nanosecond-gated, image-intensified CCD, a significant improvement on all previous shutter implementations. Overall, the TGL microscope was demonstrated to strongly suppress prompt fluorescence by a factor of 5500:1 and reportedly achieved a detection efficiency of 42%. Electronic switching of the excitation and detection pathways enabled the gate delay to be varied from 0−200 μs in 10 μs increments, an important capability considering $\tau = 46\,\mu s$ for Pt complexes.[13] While the instrumentation was innovative, the luminophores were arguably the weak link in the system. Pt-porphines are quenched by oxygen, and their luminescence lifetimes are dependent on oxygen pressure in the sample. Moreover, the

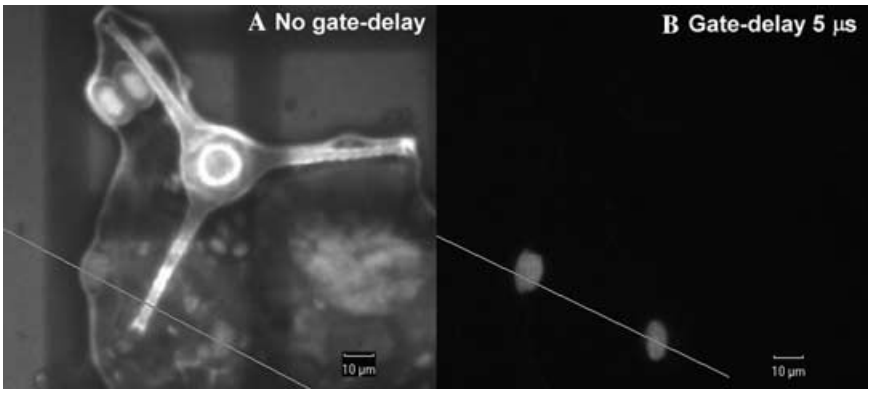

FIGURE 4. High-power UV light emitting diodes (LEDs) have recently become available with the capacity to deliver more than 400 mW of pulsed light at 365 nm. The two *Giardia* cysts located adjacent to the spines of the autofluorescent desmid seen here were labeled with the europium chelate BHHST. Prompt fluorescence from the desmid spine was reduced 25-fold due to the rapid LED switch-off that permitted gate-delay to be reduced to 5 μs.

complexes had a low quantum yield of 0.05 to 0.1 and were relatively difficult to excite at their secondary absorption peak.

Light Emitting Diodes

Light emitting diodes (LEDs) have improved steadily since the introduction of the first commercial devices in 1962. While red, green (1968), and yellow (1978) LEDs have been available for many years, blue LEDs remained elusive. Nakamura, at Nichia Chemical Labs in Japan, finally developed blue and green heterostructure LEDs with efficiencies approaching 10% in 1993. Nichia announced the first UV emitting LED in 1997 and released the world's first high-power UV LEDs (100 mW) in 2002. Currently available (single die) UV LEDs can deliver 270 mW of UV at 365 nm in CW mode, or 500 mW in pulsed mode. The output wavelength of 365 nm is longer than ideal for lanthanide chelates, however absorption is still around 40% and effective excitation is achieved due to the high optical power. The UV output of the LED can be switched very rapidly (ns), but the devices also emit luminescence broadly centered at 550 nm that persists for tens of microseconds. This emission is of low intensity and can easily be filtered out.[32] LEDs have the advantage that they can be driven at low voltage with simple electronics and their small size permits mounting of the device within the filter assembly of the microscope.[28]

UV LEDs versus Flashlamp Excitation

It is interesting to compare SNR achieved using a flashlamp-excited TGL microscope (see FIG. 3) versus images obtained with an instrument employing UV LED excitation (FIG. 4). The *Giardia lamblia* cysts were both labeled with the same europium chelate and images were acquired on the same instrument using a gated image intensified CCD detector. The rapid LED switch-off permitted the gate delay to be reduced to 5 μs instead of the 60 μs required when using the flashlamp. With UV LED excitation, 8-bit monochrome images were acquired with reduced background (8 versus 17) and the SNR was superior (25-fold versus 11-fold improvement) compared to that of FIGURE 3. Driving the LED at 400 Hz also enabled a 15-fold improvement in image capture speed (0.5 s versus 7 s).[28]

Detectors

Unlike photomultipliers or avalanche photodiodes, conventional CCDs cannot amplify the number of electrons generated from each photon impact. They can however integrate charge for several minutes when cooled to minimize thermally induced noise. Luminescence output from TGL microscopes is reduced in comparison with conventional fluorescence microscopy and it is often necessary to integrate the faint emission over many TGL cycles to improve the SNR. Even with more efficient luminophores and excitation, the flashlamp-excited TGL microscope recently (2003) reported by Soini and colleagues still required a 60-second integration period to adequately capture delayed luminescence images on a cooled CCD.[24] CCD sensors implement an electronic shutter by shunting all photon-induced charge to ground, but this technique can only be used to delay an exposure. Activating the shutter once the sensor has acquired an image will result in its erasure and cannot therefore be used to gate

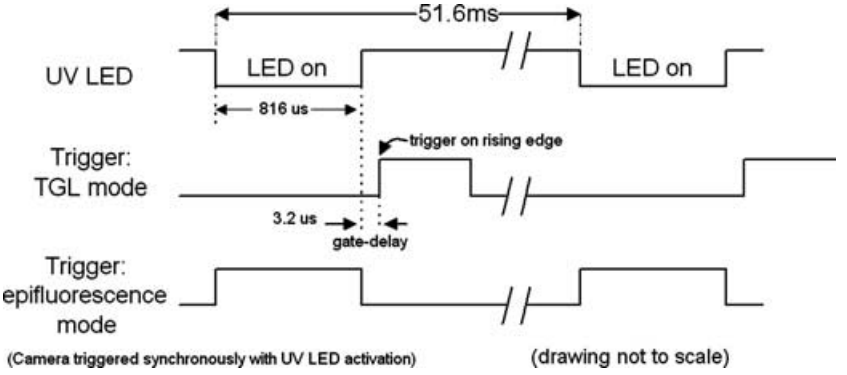

FIGURE 5. The EMCCD-equipped TGL microscope could be switched from prompt (epifluorescence) mode to TGL mode by changing the time at which the trigger pulse was sent to the camera. In prompt mode, the camera was triggered at the same time the LED was driven on. In TGL mode, the trigger was delayed until the LED had extinguished and all prompt fluorescence ceased, the camera was then triggered to capture persistent luminescence.

the sensor for the purpose of integrating emission over multiple excitation cycles.

Image-Intensified Gated CCD

A micro-channel plate (MCP) image intensifier can be visualized as an array (typically 1024×1024) of miniature photomultiplying channels each about $10\,\mu m$ in diameter. The array is terminated with a fluorescent target and the resultant intensified image is then optically coupled to a CCD detector. A gating electrode is used to rapidly switch the MCP into conduction, delivering photo-multiplication gains of 10,000 or more. This composite assembly provided a means to amplify photon-induced charge and to gate the detector with nanosecond resolution.

Although expensive, MCP image intensifiers offered significant benefits for TGL microscopy, and following the report of Hennink in 1996, many TGL microscopes were similarly equipped.[9,13,16,33] MCP image intensifiers have low quantum sensitivities of around 20%, so while gain is high, most incident photons will not be detected. When operated at high gain, the image appears grainy (night-vision effect) and the dynamic range is limited. Further, the image intensifier can be damaged when driven incorrectly.

Electron-Multiplying CCD

An invention with great relevance to TGL microscopy was filed with the U.S. patent office by Jaroslav Hynecek in 2001 describing "... a solid-state image intensifier fabricated in a monolithic form on a single piece of a semiconductor substrate."[34] CCD sensors exploiting this effect were manufactured by Texas Instruments (Dallas, TX) under the trade name "Impactron" and initially offered moderate gains of about 100-fold. Newer devices were released in 2004 with gains up to 1000, and the most recent sensor modules are stated to deliver absolute gains of up to 2000. Unlike complex MCP image intensifiers, solid-state monolithic image intensifiers can be mass produced on a silicon wafer to achieve large economies of scale. Quantum efficiency of impactron devices is high (~70% at 650 nm) compared with MCP intensifiers.

UV LED Excited TGL Microscopy with Electron-Multiplying CCD Detection

The basic requirements for TGL microscopy are relatively straightforward, a pulsed excitation source and a gated detector. As technologies with application to the field have appeared, they have been promptly applied: FELC shutters, AOM laser and gated MCP image-intensified CCDs. The great utility of UV LEDs to TGL applications has similarly been recognized; these devices have the requisite power and pulse profile to make them near ideal excitation sources for lanthanide chelates. Impactron CCDs represent a platform technology with important ramifications for TGL microscopy. Electron-multiplying (EM) CCDs have sufficient sensitivity and gain to capture delayed luminescence images in *real-time* when used in conjunction with suitable excitation sources. In this section, we describe how a commercial fluorescence microscope may be equipped with an EMCCD camera and compare the performance with earlier technologies.

Practical TGL Microscopy Using EMCCD Detection

An Andor iXon EMCCD camera (Andor Technology, Belfast, Northern Ireland) was mounted on an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) that had been converted to use UV LED excitation.[28]

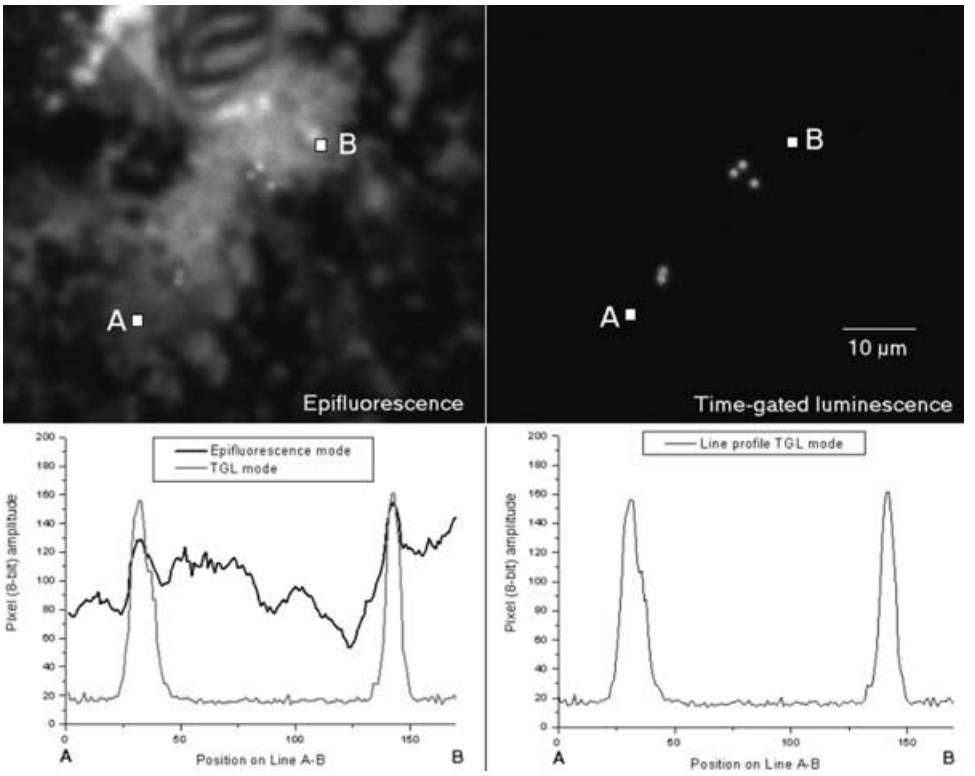

FIGURE 6. Phycoerythrin-rich algae were obtained from the University lake and spiked with 1 µm europium polymer microspheres. Slides were examined in conventional epifluorescence mode and TGL mode using UV LED excitation and EMCCD detection. Autofluorescence from the algae was strongly suppressed by a factor of 13.8, greatly increasing the visibility of the microspheres. Frame acquisition was achieved following a single excitation cycle of 500 µs and exposure of 3 ms. The line profiles below the images report pixel intensities along an (invisible) line drawn between the points A and B for prompt and TGL modes. The leftmost frame includes both profiles to show the extent to which non-specific autofluorescence has been suppressed.

FIGURE 5 shows timing of the UV LED pulse in relation to the trigger pulse sent to the EMCCD camera. The camera is operated in "fast external trigger" mode and responds within nanoseconds to the rising edge of the trigger pulse to capture and store a frame. A new frame can be captured every 33 ms, although the LED pulse frequency was maintained below this repetition rate. The sensor is erased prior to each capture, thus only phosphorescence arising from a single TGL cycle is captured every frame. A microcontroller was used to drive the LED and to trigger the EMCCD camera after a period of 3.2 µs. This short gate-delay results in negligible loss of luminescence signal from the target luminophore that has a lifetime in excess of 300 µs. Luminescence decay of most lanthanide chelates follows single exponential kinetics, so this brief delay corresponds to a reduction in initial luminous intensity of less than 1%. The instrument can be used in conventional fluorescence mode or switched (instantly) to TGL mode as required. In conventional fluorescence mode the LED was driven at reduced current (200 mA, 500 µs pulse) and the camera triggered simultaneously with the LED at the natural refresh rate of the camera (30 Hz). In TGL mode, the LED was driven hard (1 A, 500 µs pulse) at the same 30 Hz refresh rate, but the camera was not triggered until the LED had extinguished. This configuration delivered real-time images to the screen in either conventional fluorescence mode or in TGL mode.

Results: UV LED Excitation and EMCCD Detection

To test the ability of the instrument to suppress prompt autofluorescence, we isolated phycoerthrin-rich algae from a nearby lake and spiked the sample with 1 µm europium polymer microspheres. The mixture was first examined in conventional epifluorescence mode and images were then acquired in TGL mode as shown in FIGURE 6. Autofluorescence from the algae was strongly suppressed by a factor of 13.8,

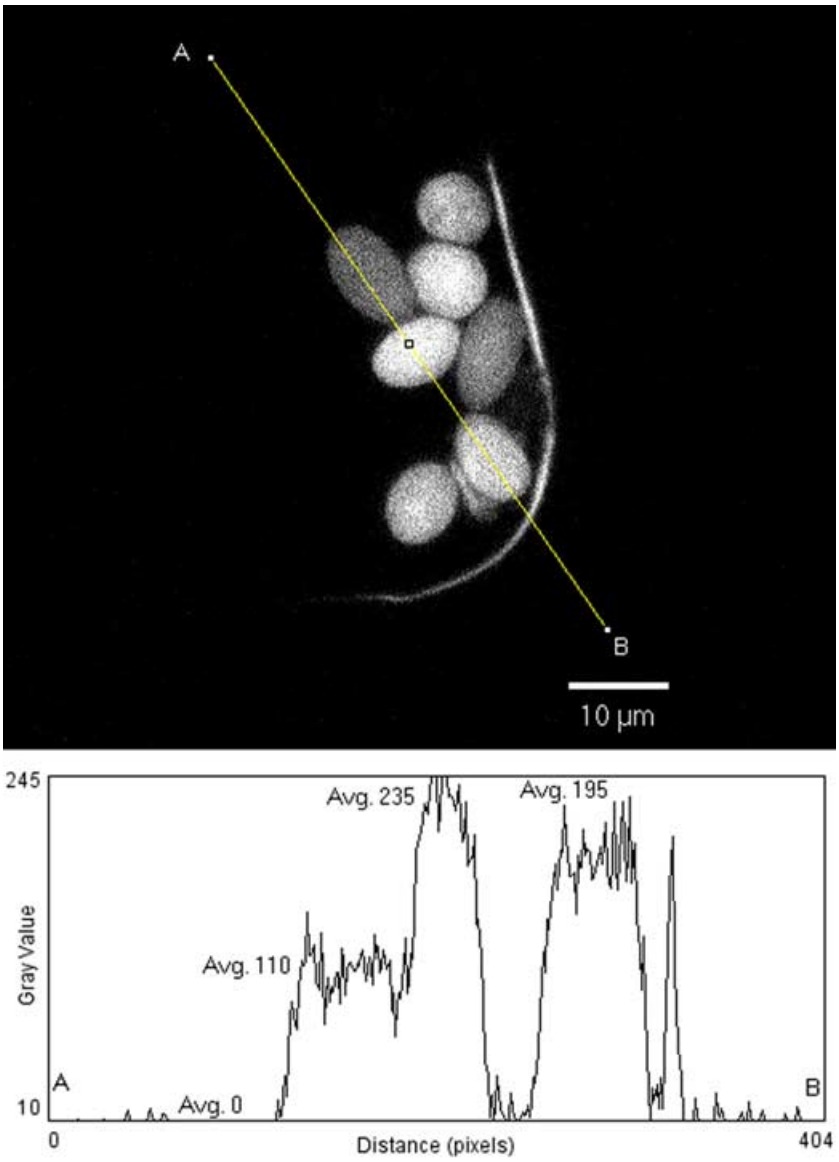

FIGURE 7. *Giardia lamblia* cysts were labeled with the europium chelate BHHST and analyzed in TGL mode using the UV LED-excited EMCCD. The LED excitation pulse was 500 µs, emission was collected for a period of 6 ms, and neither binning nor frame averaging were enabled. Gain of the EMCCD camera was 35, a minimal value considering the maximum gain setting for the camera was 4096. The image shown here is the result of a single TGL cycle that could be repeated every 33 ms to permit real-time TGL operation.

greatly increasing visibility of the microspheres. Frame acquisition was achieved at a gain of 400 with a short 4 µs gate delay, a single excitation pulse of 800 µs and exposure of 3 ms.

Immunofluorescently Labeled Pathogen

The europium microspheres imaged against the autofluorescent background shown in FIGURE 6 were intensely luminescent and easily resolved by the EMCCD camera in TGL mode. It was necessary then to evaluate the detection capability of the instrument when presented with weaker signals, as for example with immunofluorescently labeled cells.

A pure suspension of *Giardia lamblia* cysts was labeled with G203-BHHST immunoconjugate[28] and viewed under TGL conditions using UV LED excitation and

EMCCD detection. For the image shown in FIGURE 7, the camera gain was set at 35, exposure at 6 ms, and binning of adjacent pixels and frame averaging were not used. A line profile sampling the pixel values across the region is represented by the line marked A-B superimposed on the captured image. The average pixel value for the line as it transects each of the three cysts is shown in the plot below the figure. This monochrome image represents each pixel as an 8-bit value; white (255) and black (0). The cysts were efficiently labeled and could be detected faintly even without electron multiplication in TGL mode. The excellent resolution achieved here relied greatly upon three factors: the intensely luminescent chelate (BHHST), the powerful excitation source that terminates within microseconds, and the high-gain EMCCD camera.

Conclusion

The pace of development in TGL microscopy has not slowed since Soini expressed the desire in 1988 to "... re-test the *old idea* of time-resolved fluorescence microscopy in immuno-histology and cytology." The key advantage of TGL microscopy is its ability to greatly reduce image complexity. Frames are devoid of content (featureless black) unless a target particle is present, thus most images can be processed at high speed using simple image-processing algorithms. The time required to acquire a delayed luminescence image has steadily decreased over the last two decades as a consequence of the application of new technologies. With the EMCCD system described here, it is possible to scan a slide and process the images in *real-time* (20 frames a second). Frames with putative target can be marked for more detailed analysis using other modalities such as differential interference contrast or secondary fluorescent labels to increase confidence. In this manner an automated scheme can be implemented where a microscope/computer workstation scans multiple slides at high speed, marking the location of potential positives for later detailed review by an expert-system or human operator.

Conflict of Interest

The authors declare no conflicts of interest.

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