Measurement of action spectra of light-activated processes

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Abstract. We report on a new experimental technique suitable for measurement of light-activated processes, such as fluorophore transport. The usefulness of this technique is derived from its capacity to decouple the imaging and activation processes, allowing fluorescent imaging of fluorophore transport at a convenient activation wavelength. We demonstrate the efficiency of this new technique in determination of the action spectrum of the light mediated transport of rhodamine 123 into the parasitic protozoan *Giardia duodenalis*.
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1 Introduction

Light sensitivity of organisms is instrumental in avoiding damaging UV radiation (through phototaxis)[1–4] or to maximize the amount of energy extracted from the light source (through phototropism and photosynthesis).[3,5–8] The spectral response of these processes is of vital importance for understanding the underlying mechanisms of the interaction.

The interaction of light with some organisms also drives the transport of solutes across the cellular membrane. For example, in the archaeon *Halobacterium salinarium*, there are at least two light-mediated membrane transporters, both based on a rhodopsin species.[9,10] Bacteriorhodopsin pumps protons

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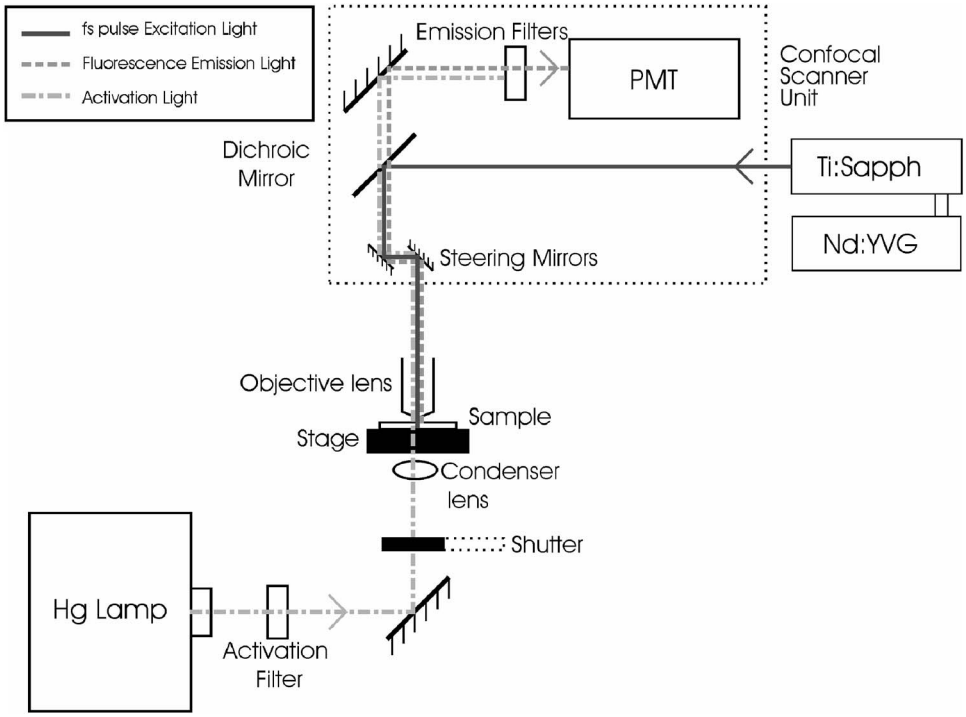

Fig. 1 Schematic of apparatus showing optical paths. Solid line: femtosecond (fs) pulse excitation light; dash-dot line: activation light from mercury (Hg) lamp; dashed line; fluorescence emission light. PMT: photomultiplier tube.

and halorhodopsin drives a chloride pump.[3] The transport of these solutes may be measured by patch clamping methods[11,12] or alternatively by optical microscopy. Imaging of calcium ions has been conducted using two-photon excitation at an electronic resonance of the calcium ion, and monitoring the fluorescence emission of the ion.[13,14]

In many instances, monitoring of the transport of the fluorophore into the cell using fluorescence microscopy or single-photon confocal microscopy is problematic. This is because of the dependence of both the process itself (activation) and the imaging (excitation) on the wavelengths being used. As the excitation wavelength is tuned away from the optimum wavelength of the fluorophore, there is less fluorescence emission, thus decreasing the signal-to-noise ratio of the image. For excitation wavelengths longer than the emission wavelength of the fluorophore, there is virtually no fluorescence emission,[15,16] making the tracking of the fluorophore almost impossible.

Quantifying light-activated transport of a fluorophore using full-field microscopy, such as epifluorescent bright field microscopy, suffers from the problem that fluorescence from outside of the image plane is also collected.[17] This results in higher than actual fluorescent intensities being recorded from the regions of interest. Thus, for accurate quantification of fluorophore transport, ideally confocal microscopy should be used.

In the parasitic protozoan *Giardia duodenalis*,[18,19] a light-mediated transporter exists that pumps the fluorophore rhodamine 123 into the cell. This effect has been observed to occur in response to high-fluence blue-light illumination.[20] Our preliminary measurements showed that the transporter is not excited by two-photon absorption of light centered around 800 nm (data not shown).

We have developed a hybrid of single-photon and multiple-photon imaging techniques that is able to decouple the activation of the membrane transporter and the excitation of fluorescence from the fluorophore.

2 Experimental System

The system consists of two separate illumination systems, the imaging system (solid line) and the activation system (dash-dot line) (Fig. 1). The imaging system consists of an upright microscope (BX-50, Olympus, Tokyo, Japan) attached to a confocal scanning control box (Fluoview®, Olympus), which was modified in-house for two-photon microscopy. Images are collected using a 60×/1.40-NA Planapo oil immersion lens (Olympus). A 532-nm±5 nm (CVI, Albuquerque, NM) bandpass filter prior to the photomultiplier tube (PMT) isolated fluorescence emission light from activation or excitation light. Femtosecond laser pulses for two-photon microscopy are generated by a mode-locked Ti:sapphire laser (Mira-900F, Coherent, Santa Clara, CA) pumped by the second harmonic from a cw Nd:YVG laser (Coherent Verdi). The femtosecond laser was tuned to 800 nm with a spectral bandwidth of 12 nm. The optical system provided an optical sectioning thickness of $\sim 1\ \mu$m.

The activation system consists of a mercury lamp (Olympus U-LH100HG 100 W) spectrally filtered using a set of bandpass filters. The selected spectral portion is coupled to the optical train of the microscope and activates the sample through a condenser lens. The power of the incident light was

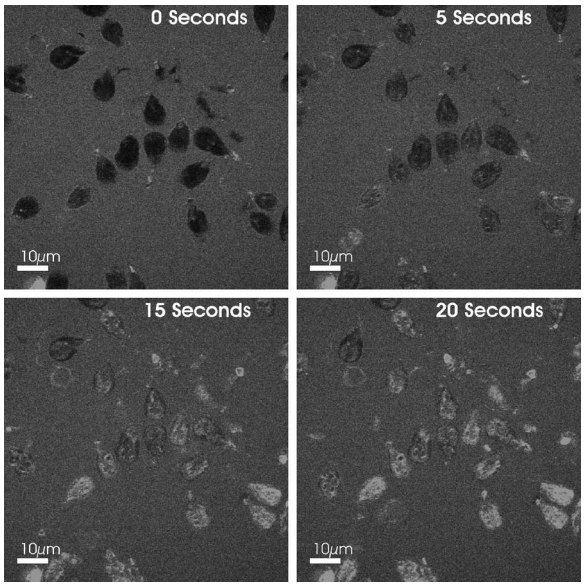

Fig. 2 Time series of light-induced uptake of rhodamine 123 by *G. duodenalis*. Image was acquired using the system described in the text. Activation wavelength and irradiance was 380 nm and 92.3 μW/mm^2, respectively.

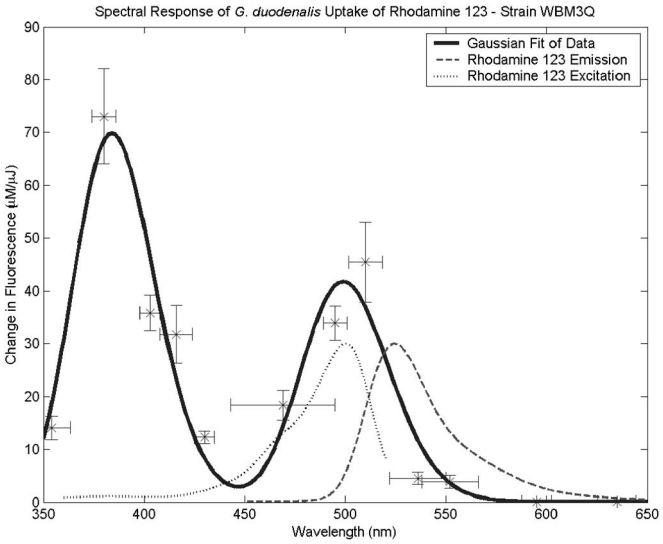

Fig. 3 Spectral dependence of light-induced uptake of rhodamine 123 by *G. duodenalis*. The crosses represent the center wavelength of the activation filter. The solid lines are Gaussian fits to the data (calculated in frequency space). Dotted and dashed lines are normalized excitation and emission spectrum of rhodamine 123, respectively. The standard error of the mean and full width at half maximum of the relevant filter is represented by vertical and horizontal error bars, respectively. Each point has been normalized for incident power and is an average of at least three independent assays, each consisting of more than 20 cells. Rhodamine 123 excitation and emission spectra are measured in phosphate buffered saline.

maintained to be approximately equal at each recorded wavelength by the appropriate choice of neutral density filters. Attenuation values for each bandpass filter were predetermined by the calibration procedure, which involved measurement of both the throughput illumination power and irradiance (W/cm^2). This was done by measuring the power through a 100-μm-diam pinhole at the sample plane. Images were acquired using Fluoview software and analyzed using Matlab.

This system allows simultaneous and independent activation of the light-induced membrane transport and imaging of the fluorophore. For activation wavelengths that fall within the excitation range of rhodamine 123, sequential activation and imaging was used, i.e., the sample was illuminated with activation light while an image was not being acquired, and the activation light was blocked while an image was acquired. Synchronization was achieved between activation and imaging through the use of a shutter before the condenser lens (Fig. 1) to block activation light from reaching the sample. The activation time for sequential and simultaneous imaging was 3.7 and 5 s, respectively. The time to acquire an image was 1.3 s.

Giardia duodenalis strain WBM3Q was axenically cultured as previously described.[21] Trophozoites were washed once in phosphate buffered saline. Rhodamine 123 (Molecular Probes, Eugene, OR) dissolved in H_2O was added to a final concentration of 30 μM. Wet-mounted slides were used immediately.

3 Results

The imaging system described before was used in the measurement of the spectral photosensitivity of the optically induced transport of rhodamine 123 into *G. duodenalis* trophozoites. Figure 2 shows a time series of images of uptake of the fluorophore. The concentration of fluorophore inside the trophozoites increases dramatically and can become significantly higher than that of the surrounding medium. As a result of the light-activated pumping action of the cells, almost all of the dye in the solution is pumped into the cells, so that the background fluorescence essentially vanishes. The fluorophore accumulation in the cells around the two nuclei and in small vesicles in the dorsal region of the trophozoite is clearly observable.

The use of two-photon fluorescence imaging allows decoupling of the imaging excitation and the activation of the fluorophore transport, so that the spectral response can be acquired over the full spectral range of interest. In single-photon microscopy, the excitation wavelength used must be within the excitation region of the fluorophore, only allowing wavelength scanning within that spectral portion. For rhodamine 123, this would be from 460 to 520 nm (Fig. 3). Investigation of the action spectrum outside of this region is problematic because of the lack of fluorescence emission.

Using our new technique, the spectral response of the light mediated transport of rhodamine 123 by *G. doudenalis* has been measured (Fig. 3). Each point represents one bandpass filter. The activation spectrum consists of two large absorption peaks, centered at wavelengths of 381 and 502 nm, respectively. The Gaussian peak fit was carried out in frequency space using OriginPro 7.5. The main cause of the uncertainty in the measurements is the variation of individual cells' responses. This is represented by vertical error bars in Fig. 3. This action spectrum is significantly different from the excitation spectrum of rhodamine 123 (Fig. 3, dotted line).

4 Conclusion

The investigation of light-mediated cellular transport using single-photon fluorescence microscopy can be inherently difficult in regions of the spectrum where the fluorescence quantum efficiency of the fluorophore is low. By separating the imaging and activation systems, we develop a technique that permits the imaging of cellular transport at any activation wavelength. This allows the collection of the entire action spectrum of a light-mediated transport process using one apparatus. Investigation of such a process using only one incident light source is not possible over such a large spectral range, because the image acquired relies on the fluorescence emission of the fluorophore which (for rhodamine 123) decays rapidly as the wavelength is shifted toward the UV or to wavelengths higher than the maximum emission wavelength. As such, using two-photon excitation confers several advantages, most importantly decoupling the imaging and activation processes. Thus, the action spectrum may be determined over the entire spectral range independent of the fluorophore used. Secondly, by virtue of the important property of two-photon confocal microscopy to detect fluorescence signal only from a thin (several microns) slice of the sample, unwanted out-of-plane background fluorescence is efficiently discriminated, yielding a high signal-to-noise ratio.

The action spectrum of transport of rhodamine 123 into *G. duodenalis* is determined using the system outlined. The resultant spectrum consists of two main peaks, one at 381 and one at 502 nm. From the acquired action spectrum, a spectral tag that can be used to identify the moiety responsible is determined, allowing it to be tracked during purification.

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