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Applying Multiphoton Imaging to the Study of Membrane Dynamics in Living Cells

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Abstract

The endomembrane system of a cell is a highly dynamic, ephemeral structure that is difficult to visualize. Reconstructions from sections of fixed material can provide high-resolution information on intercellular membrane architecture, but such techniques are fraught with artifacts and are of little help in understanding the dynamics of intracellular membrane traffic. Recently, the availability of fluorescent membrane probes and the development of techniques for optically sectioning intact specimens have allowed glimpses of membrane dynamics to be visualized in living tissue. In this review we discuss the potential of a new optical sectioning technique, multiphoton imaging, for visualizing membrane dynamics in living cells. Multiphoton microscopy offers an unparalleled ability to obtain images from deep within specimens while minimizing the effects of phototoxicity.

Keywords

cellular dynamics; imaging; membrane; microscopy; multiphoton; optical sectioning; two-photon

Visualizing the three-dimensional structure of membranes within cells is often a formidable challenge. The endomembrane system of a cell is structurally complex and highly dynamic. When a cell divides the endoplasmic reticulum, nuclear envelope and Golgi are dispersed into vesicles at mitosis and reformed at telophase. Even in nondividing cells there is a complex, highly orchestrated traffic of vesicles transiting the secretory pathway. Specimens may be fixed and sectioned so that the membrane organization at a specific instant of time can be inferred from three-dimensional reconstructions of serial sections. Unfortunately, most fixation protocols for light and electron microscopy do a poor job of preserving membrane structure, often giving rise to significant artifacts. More importantly, reconstructing dynamic events from random snapshots of different cells is an extremely difficult undertaking. These problems can, in principle, be avoided when live specimens that have been labeled with specific membrane probes are observed. Although live imaging precludes the use of electron microscopy to reveal ultrastructure, this strategy nevertheless can yield valuable information on membrane organization and can complement ultrastructural studies. However, live specimens are generally of poor optical quality. Microinhomogeneities in refractive index caused by cytosol/membrane interfaces scatter

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light and degrade image quality, especially when viewing structures deep within the specimen.

If membranes are labeled with fluorescent probes the image is also blurred by out-of-focus flare from fluorescence emission emanating from above and below the plane of focus. This problem is particularly apparent when observing membranes because of their extended, convoluted laminar structure. There are several ways of optically sectioning a specimen and thereby eliminating out-of-focus interference, including image deconvolution (1), confocal imaging (2) and multiphoton imaging (3).

Optical Sectioning by Image Deconvolution

This technique uses images from a standard, wide-field fluorescence microscope with all the attendant advantages (low cost, use of broad-band excitation sources such as mercury arcs that can excite a wide range of fluorophores and use of high sensitivity charged coupled device (CCD) imaging detectors). A focal series of images is collected, digitized and processed by a computer algorithm that uses a knowledge of the point-spread function of the microscope to deconvolve and hence restore the images. This technique works well with relatively thin, fixed specimens with stable fluorophores that allow the collection of images with high signal-to-noise ratios. In these situations, deconvolution microscopy can both de-blur an image and also provide a valuable increase in resolution. However, image restoration by computational deconvolution is often not so successful with live specimens. Any movements that occur in the specimen while a stack of optical sections are being recorded will abrogate successful deconvolution, as will images from faint sources that have poor signal-to-noise ratios, such as those typically obtained from living specimens.

Optical Sectioning by Confocal Microscopy

In most commercial realizations of this technique the fluorophore excitation source is a laser beam that is scanned over the specimen. The fluorescence signal traverses the reciprocal path of the excitation, is thereby descanned and then filtered by a pinhole aperture before passing to a photodetector. The pinhole is situated in a conjugate focal plane to the focal plane being observed in the specimen. Fluorescence emitted from the immediate vicinity of the point being illuminated by the focused laser beam passes through the pinhole unimpeded. However, fluorescence originating from above and below the plane of focus will be spread out and thus be largely blocked by the aperture. In this way, the aperture effectively suppresses out-of-focus interference, thereby producing a clean image of the optical section.

Confocal microscopy is an effective tool for *in vivo* imaging of membrane dynamics and is often used in this application (4). However, this technique does have limitations. In common with wide-field fluorescence microscopy, the whole volume of the specimen is illuminated. Excitation of fluorophore outside of the focal plane is unavoidable. Unfortunately, it is also undesirable because any fluorophore excitation produces a significant proportion of nonradiative transitions to the ground state that can generate reactive products, such as singlet oxygen, which are toxic to the cell and give rise to fluorophore bleaching. In the case

of a typical specimen with a thickness of 20 μm and an optical section thickness of 0.5 μm , only around 2.5% of the excitation is needed for imaging; the remainder creates interference that has to be eliminated with the confocal aperture. In addition, fluorophore bleaching with the attendant toxic effects still occurs in this nonimaged region.

Optical Sectioning by Multiphoton Microscopy

This comparatively new laser-scanning microscopy technique uses a nonlinear optic effect in order to confine fluorophore excitation to the focal plane under observation. In so doing, several problems associated with wide-field fluorescence microscopy are eliminated. Interference by out-of-focus fluorescence is avoided simply because this fluorescence is never generated in the first place. Similarly, toxic effects arising from excitation of fluorophore outside the optical section being observed are avoided because only fluorophore in the optical section is excited.

Confinement of excitation to a single focal plane is achieved by multiphoton excitation. In the case of two-photon microscopy (most commonly used) fluorophore excitation within the bulk of the specimen is avoided by using an excitation wavelength of around twice the excitation peak of the fluorescent probe being used. Typically, the excitation spectrum of a fluorophore is sufficiently narrow that there will be essentially no fluorophore excitation by this longer wavelength of light. However, if the photon density is sufficiently high, there is a significant probability that two photons will be absorbed simultaneously by a fluorophore, effectively behaving as a single photon with an energy equal to the sum of the energies of the two individual photons. As photon energy is inversely proportional to wavelength, this will be equivalent to absorbing a photon at the excitation maximum for the fluorophore, thereby producing fluorescence. The power density required for two-photon excitation is very high – in the order of 10^{21} watts/cubic meter. Such a power density may be achieved in the focal volume of a high numerical aperture objective. However, if delivered continuously this power level would severely damage living specimens. By using ultrafast, pulsed lasers it is possible to attain the required peak powers within the duration of a very brief pulse yet maintaining mean power levels that are benign to living specimens. Typically, mode-locked Ti:sapphire lasers are used as excitation sources that produce pulses of 100 femto-seconds at a repetition rate of 80MHz and power levels of around 10 mw at the objective.

Multiphoton effects are intrinsically nonlinear. In the case of two-photon excitation, fluorescence varies as a quadratic function of intensity. Similarly, with three-photon excitation, fluorescence varies as a cubic function of intensity. In a multiphoton laser-scanning microscope, the power density is only sufficient to support multiphoton excitation at the focal volume of the objective lens. Away from this region of excitation the beam is either converging or diverging and power density drops off rapidly, essentially eliminating any excitation away from the focal volume. A significant secondary advantage of multiphoton imaging is that the optical configuration of a multiphoton microscope can be made simpler than that of a confocal microscope because the signal does not have to be de-scanned and focused onto a pinhole. This leads to improvements in optical performance as described below.

The resolution of a multiphoton microscope is similar to that of a confocal microscope. This at first sight is surprising as the resolution is defined by the geometry of the focal volume of the excitation which, in the case of two-photon microscopy, is twice the wavelength of that used in a confocal microscope. However, because the excitation is proportional to the square of the power-density, the point spread function is narrowed by squaring, which almost compensates for the wavelength increase. In practice, there is rarely any observable difference in resolution between a confocal and a multiphoton microscope.

The main advantages to the biologist of multiphoton microscopy compared to other optical section strategies are an unparalleled ability to obtain images from deep within a specimen and a significant reduction of phototoxicity for large specimens. The disadvantages are primarily high cost, problems with damage caused by single-photon excitation in some specimens (typically highly pigmented specimens) and higher levels of in-plane bleaching (see below) than in a confocal microscope.

Obtaining Images from Deep Within Living Tissue Using Multiphoton Microscopy

There are three main reasons why multiphoton microscopy can usually obtain images from deeper within specimens than either confocal or wide-field microscopy:

1. In a multiphoton microscope, fluorophore excitation only occurs at the plane of focus. Any fluorophore situated above this plane is therefore not excited and does not absorb and attenuate the excitation beam.
2. The longer wavelengths used for excitation are less scattered than those used for single photon excitation. Light scatter is a significant problem when imaging live specimens because of microinhomogeneities in the refractive index of the cytosol. Scattering is highly wavelength dependent; for example, Raleigh scattering varies as the inverse fourth power of wavelength, and thus longer wavelengths tend to scatter less.
3. The resolution of a multiphoton microscope is defined by the point-spread function of the excitation focal volume; i.e., fluorescence is only generated at the focus of the excitation beam. Photons emitted from the volume may be scattered; however, as long as they find their way to the detector, they can be used for imaging. This contrasts with the situation in confocal microscopy where, if a photon emanating from the focus of illumination is scattered, it will not pass through the confocal pin-hole. On the other hand, with confocal imaging some photons from out-of-focus regions may be fortuitously scattered onto a trajectory emanating from the point of focus, and therefore pass through the pin-hole and contribute to an unwanted background signal.

For the reasons stated above, multiphoton imaging can obtain images from deep within specimens, whereas images obtained at the same depth in a confocal microscope are often significantly degraded (Figure 1). The improvements are specimen dependent but typically are around a factor of four (5). The differences between the two imaging modes are often

more striking when membranes are being visualized in living specimens. Living specimens are rather turbid optically because of light scattering at cytosol/membrane interfaces and cytoplasmic granules. In addition, many membrane probes label all membranes within a cell, leading to marked excitation extinction effects in confocal microscopy. In some applications, such as studies of brain-slice preparations (6) or developing embryos (7), the increased depth penetration capabilities of multiphoton imaging can offer critical advantages.

Potential for Improved Viability with Multiphoton Imaging

The confinement of fluorescence excitation (and hence toxic product generation) to a single focal plane offers the prospect of a considerable reduction in the generation of toxic products in live specimens, but has the disadvantage that bleaching at the focal plane may be greater than when using single photon excitation. It has been reported that in the case of two-photon excitation (square-power law) in-plane bleaching rates may vary as a third or even higher power of excitation, depending on the fluorophore (8). These observations suggest that excitation levels should be kept as low as possible, consistent with obtaining sufficient signal, and that viability improvements with multiphoton imaging may not be realized with thin specimens such as cultured cells. The improvements are likely to be dependent on the specimens being observed. For example, bigger improvements can be anticipated when observing embryos of 100 μm thickness compared to single cells with a thickness around 5 μm . In a study of mitochondrial dynamics in hamster embryos (Figure 2) confocal imaging was not appropriate for studying the early divisions of the living preimplantation embryos (9). Attempts to do so produced developmental arrest. However, embryos had unimpaired viability when imaged with two-photon excitation at 1047 nm (Figure 2G). Indeed, an ultimate test of viability was made by re-implanting an embryo that had been imaged every 15 min for 24 h into a pseudopregnant female and recovering a healthy hamster pup (Figure 2H). Interestingly, further investigations revealed that the excitation wavelengths used for confocal imaging (514 nm, 532 nm or 568nm) were toxic to the embryos (9). The 1047 nm excitation used for multiphoton imaging appeared to be completely benign to the embryos, even though the mean excitation powers were around 1000-fold higher than those used for confocal imaging.

Studying Membrane Dynamics with Multiphoton Imaging

The increased depth at which images can be acquired afforded by multiphoton imaging has proven to be a powerful tool for the study of membrane dynamics in thick specimens such as *Caenorhabditis elegans* embryos. An early embryo is bathed in a medium containing a lipid-permeable membrane probe, and the eggshell permeabilized with a laser microbeam in order to allow ingress of the probe (Figure 3). The plasma membranes of all the cells in the embryo are rapidly labeled, extending into the internal endomembrane system, thereby allowing the observation of endocytic events (Figure 4). In a similar manner it has been possible to study membrane fusion (10) and the membrane trafficking events that occur during the final scission of the residual intercellular canal during cytokinesis (11).

In practice, we find that multiphoton imaging gives better signal-to-background ratio images than confocal imaging, even when specimens are not thick enough for the deeper penetration

capability of multiphoton imaging to be apparent ($<20\ \mu\text{m}$). This is probably because it is usually necessary to use wider pin-hole apertures in a confocal microscope than is optimum for resolution in order to obtain a stronger (i.e. less noisy) signal. In contrast, a multiphoton microscope has a simpler, more efficient optical path for emission and has no confocal aperture.

Typically, single-beam laser-scanning microscopes acquire a 512×512 pixel image in 1 s. Resolution can be traded off for speed when viewing rapid processes. This speed may be insufficient to observe very rapid processes such as vesicle fusion; nevertheless, promising advances have been made using multiphoton microscopy to study rapid processes such as membrane fusion/fission events (Figure 4), changes in the cytoskeleton, or pH and calcium fluxes (12–16).

Image acquisition speed is ultimately limited by fluorophore saturation. Faster frame rates result in shorter pixel dwell times, causing fewer photons to be detected at each pixel, thereby degrading the signal-to-noise ratio of the acquired images. The lower signals produced by faster frame rates can only be compensated by increasing excitation up to the onset of fluorophore saturation (caused by ground-state depletion). Increasing excitation beyond this point gives no increase in signal and reduces signal-to-background ratios. Unfortunately, laser-scanning microscopes operate at excitation levels that are not far from fluorophore saturation, so there is little hope of increasing speed by using higher levels of excitation. Indeed, the observation of the high power-law dependence of fluorophore bleaching on excitation for multiphoton imaging (8) suggests that multiphoton microscopes should be run at low levels of excitation.

One possible way of breaking the speed barrier for laser-scanning microscopy imposed by fluorophore saturation is to use multiple parallel beams or an extended slit as an excitation source. Each beam can be of similar intensity to a single beam in a conventional system, allowing potential speed increases equivalent to the number of parallel beams. Such systems have been described for confocal microscopy (17) and recently for multiphoton microscopy (18,19). However, the nonlinear nature of multiphoton microscopy has the consequence that extending the excitation from a single beam into multiple beams or a slit would require higher levels of excitation for the same levels of fluorophore excitation. This may mean that the excitation power levels required in order to obtain a useful increase in speed become excessive.

In summary, multiphoton imaging offers unsurpassed abilities to obtain high-contrast images of fluorescent probes from deep within optically turbid specimens with the minimum of photodamage. These features open up exciting possibilities for visualizing the dynamic behavior of membranes within living tissue.

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Figure 1. Illustration of the increased depth penetration achievable with multiphoton microscopy compared to confocal microscopy

Samples of acid-fucsin-stained monkey kidney were imaged at a depth of 60 μm into the sample by confocal (550 μW of 532-nm light) and by multiphoton (12mw of 1047-nm light) microscopy. Laser intensities were adjusted to produce the same mean number of photons per pixel in each image. The confocal image exhibits a significantly narrower spread of pixel intensities compared to the multiphoton image, indicating a lower signal-to-background ratio. Multiphoton imaging provides a high-contrast image even at significant depths within a light-scattering sample. Scale bar, 20 μm . Reproduced with permission from Centonze and White, 1998.

Figure 2. Minimally invasive long-term imaging of hamster embryos

Two-cell hamster embryos, labeled with the fluorescent vital mitochondrial probe, Mitotracker (Molecular Probes) were imaged over 24h with two-photon excitation (1047 nm) on an environmentally controlled stage. Selected images from such a time series are shown in A through F. The images have been pseudocolored to emphasize regional variation in pixel intensity, with red indicating high pixel intensity levels and blue indicating low pixel intensity levels. Embryos were returned to the incubator following the imaging period and their development to the morula and blastocyst stage was subsequently assessed. These two-photon imaged embryos developed in proportions comparable to their nonimaged counterparts (G), regardless of whether images were collected every 15 min or every 2.5 min over the 24-h period. The morulae and blastocysts from imaged embryos are capable of fetal development when returned to the reproductive tract of a pseudopregnant recipient female, and in one case we permitted the pregnancy to go full term, resulting in a normal, healthy baby (H). In stark contrast, similarly prepared embryos imaged with confocal microscopy (514 nm, 532 nm, or 568 nm) were unable to develop to the morula and blastocyst stage, even when imaged for as little as 8h. This developmental inhibition occurred even in the absence of a fluorescent label (G). Scale bar for A–F = 50 μ m. (Graph (G) is reprinted with permission of Nature Biotechnology © 1999. A–F and H are reprinted with permission of Squirrell and Bavister © 1999).

Figure 3. Dye loading a *C. elegans* embryo

Embryos were imaged using 900 nm light to simultaneously generate two-photon fluorescence images (B–J) and IR transmitted light images (A). At $t = 0$ (A, B), the embryo was bathed in a solution of the membrane dye FM 2–10 (Molecular Probes) at a concentration of $100 \mu\text{M}$ in egg salts, but initially the dye was excluded from the plasma membranes of the embryo (B), although there was faint labeling of the egg shell. A Coumarin 450 dye laser was used to puncture a tiny hole in the egg-shell and vitelline membrane, at the location designated by the arrow in B. The dye rapidly entered the egg shell, becoming brightly fluorescent as it encountered the membranes (C). The cell membranes became progressively stained in a graded fashion from the site of ablation (C through G). The dye remained associated with the membrane as it invaginated to form a cleavage furrow (H, I: arrowheads). A, B: $t = 0$; C: $t = 1.5\text{s}$; D: $t = 3.0\text{s}$; E: $t = 4.5\text{s}$; F: $t = 6.0\text{s}$; G: $t = 7.5\text{s}$; H: $t = 39\text{s}$; I: $t = 64.5\text{s}$; J: $t = 90\text{s}$. Scale bar $25 \mu\text{m}$.

Figure 4. Imaging endocytosis in the *C. elegans* embryo with multiphoton excitation

The plasma membrane was labeled with FM 2–10 and imaged as described above. Budding vesicles can be seen as small protrusions at the membrane (arrowheads in A). These buds can break free of the plasma membrane (white arrowhead in B, yellow arrowhead in F) and move long (white arrowhead) or short (yellow arrowhead) distances. Some membrane buds remain associated with the plasma membrane (red arrowhead). A: $t = 0$; B: $t = 2$ s; C: $t = 4$ s; D: $t = 10$ s; E: $t = 14$ s; F: $t = 20$; G: $t = 24$. Scale bar = $25\ \mu\text{m}$.