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Photoconversion using confocal laser scanning microscopy: a new tool for the ultrastructural analysis of fluorescently labeled cellular elements

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Introduction

Fluorescence immunolabeling is a commonly employed technique that facilitates protein localization, cell structure visualization, and cell interaction studies at the light microscopic level. Unfortunately, fluorescent dyes are subject to bleaching upon visualization and are, therefore, poor permanent labels (Lubke, 1993). Moreover, they are inherently incompatible with transmission electron microscopy (TEM) because of their low electron density (Lubke, 1993). These shortcomings created the need for a technique that transforms the fluorescent dye into a permanent label compatible with light and transmission electron microscopy. Maranto (1982) first elucidated such a procedure, termed photoconversion, whereby fluorescent light was used to transform Lucifer Yellow in the presence of 3, 3'-diaminobenzidine (DAB) to a stable brown, electron dense reaction product. This technique facilitates the ultrastructural analysis of photoconverted cells, thereby supporting associated light and electron microscopic studies. Although this technique has provided a methodological interface between light and transmission electron microscopy, its use of conventional widefield fluorescence microscopy (WFM) to drive the photoconversion reaction requires lengthy procedural times and limits its ability to target specific structures in a field of fluorescently labeled elements.

Advancements in targeted photoconversion have been limited to its use in generating axotomies in fluorescently labeled nerve fibers (De-Miguel, et al., 2002). Procedural times, however, have been noticeably reduced by focusing the light energy with higher magnification objectives and/or enriching the chemical reaction with oxygen (Singleton and Casagrande, 1996; Kazca, et al., 1997). Nevertheless, continual advancements in these aspects of this technique have not exploited the technological developments made in confocal laser scanning microscopy (CLSM). Specifically, confocal laser scanning microscopy offers significant improvements over conventional microscopy by utilizing high-intensity coherent monochromatic lasers to create the excitation necessary for

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fluorescence, thereby reducing the time necessary for conversion. Moreover, conventional laser scanning microscopy offers scan zoom and specific region of interest (ROI) scanning as part of its imaging capabilities, making cellular targeting possible. Consequently, we hypothesize that replacing conventional widefield fluorescence microscopy with confocal laser scanning microscopy in the photoconversion process will enable the selective targeting of fluorescently labeled structures while concurrently reducing the overall reaction time.

In this study we assessed the procedural duration and selectivity of conventional widefield and confocal microscopy-based photoconversion. This was achieved at varying magnifications for fluorescently labeled neuronal and glial elements of the central nervous system (CNS). The results of this study provide compelling evidence that confocal laser scanning microscopy-based photoconversion facilitates a reduction in reaction time while increasing target selectivity, thereby representing a major advancement over conventional widefield microscopic methods of photoconversion.

Materials and methods

Sectioning and Immunohistochemistry

Postnatal day 14 (P14) Sprague-Dawley rat pups were anesthetized and perfused transcardially with 0.1M phosphate-buffered saline (PBS). The brain was dissected and frozen in dry-ice chilled isopentane. Coronal cryostat sections were collected at a thickness of 10 μ m. Sections to be examined by conventional widefield fluorescence microscopy were collected on Superfrost (Sigma) glass microscope slides, whereas those examined by confocal laser scanning microscopy were collected on poly-L-lysine treated glass coverslips with a thickness of 0.17 mm (#1.5). Tissue sections were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1M PBS, pH 7.4, for 30 minutes. Following three rinses in 0.1M PBS, the sections were treated with 0.1M PBS containing 1% Triton X-100, 1% bovine serum albumin (BSA), and 5% normal horse serum (NHS) for 30 minutes. To label astrocytes in the rat brain with glial fibrillary acidic protein (GFAP), sections were incubated in rabbit anti-GFAP (1:500, DAKO Z0334) in 0.1M PBS with 5% NHS for one hour at room temperature. Following three rinses in 0.1M PBS, the sections were then incubated in Alexa Fluor 568 (AF568) goat anti-rabbit IgG (1:200, Invitrogen A-11036) in 0.1M PBS with 5% NHS for one hour at room temperature. Finally, the sections were rinsed three times in 0.1M PBS. Additionally, some sections were incubated in a 0.1M PBS solution containing 100 ng/mL of the fluorescent nuclear marker 4',6-diamidino-2-phenylindole (DAPI) for 30 minutes.

Photoconversion was assessed on additional sections that were taken from adult rats which received a single daily intraperitoneal injection of BrdU at the dose of 300mg/kg, for a period of three days. Brains were removed and fixed overnight in 4% paraformaldehyde with 1% glutaraldehyde in 0.1M PBS, pH7.8, at 4°C. Coronal 50 μ m thick brain sections were cut with a vibratome through the hippocampus. Sections were processed with immunofluorescent double-labeling for BrdU (Sigma, B5002) and synaptophysin (Roche, MAB5258) as previously described (Sun, et al., 2007).

Photoconversion and Microscopy

For both conventional widefield fluorescence and confocal laser scanning microscopy, the chemistry of the photoconversion procedure closely followed that outlined by Maranto (1982) and Sandell and Masland (1988). Although many fluorescent labels have been used successfully, there is no previous data concerning photoconversion for one of the fluorophores used in this study, Alexa-Fluor 568 (AF568). To determine a baseline for the conventional widefield microscope (Olympus IX70, 100W mercury lamp), the

photoconversion procedure was carried out with a new tissue section and fresh DAB solution for both the 20× (0.4 N.A.) and 40× (0.55 N.A.) objective lenses. Specifically, an immunolabeled section was pre-incubated in cold 0.1M Tris buffer, pH 8.2, in the dark for 5 minutes prior to conversion and placed on the microscope stage. Once the area to be photoconverted was selected, the buffer was replaced with a cold DAB solution consisting of 1.5mg/ml DAB in 0.1M Tris buffer, pH 8.2. The section was then illuminated with the fluorophore's absorption wavelength until fluorescence was photobleached and a substantial brown reaction product was formed, as assessed with brightfield microscopy.

The photoconversion procedure for the confocal laser scanning microscope approximated the conventional widefield approach, with modifications made to take advantage of the zoom and ROI scanning capabilities. The procedure was assessed by a Leica TCS-SP2 (AOBS) inverted confocal laser scanning microscope equipped with a 405 nm diode laser, a multi-line Ar laser (458, 488, and 514 nm), 3 separate HeNe lasers (543, 594, and 633 nm), and several objective lenses including: 20× (0.7 N.A.) dry, 40× (1.25 N.A.) oil, and 63× (1.4 N.A.) oil. This study also included use of a Zeiss LSM 510 META NLO upright 2-photon/confocal laser scanning microscope equipped with a multi-line Ar laser (458, 488, and 514 nm), a 561 nm diode-pumped solid state laser, a 633 nm HeNe laser, and several objective lenses including a 10× (0.3 N.A.) dry lens. Both confocal laser scanning microscopes were equipped with a galvanometer driven point scanning system and an acousto-optical tunable filter (AOTF) that enabled scan zoom and ROI scanning (i.e. regionally defined beam blanking), respectively. Because an inverted confocal laser scanning microscope (Leica) was used, tissue sections were collected on 0.17 mm (#1.5) thick poly-L-lysine coated glass coverslips to reduce light scatter through the glass and accommodate the working distances of the high N.A. objective lenses used, thereby minimizing spherical aberrations.

Briefly, a section was bathed in 0.1M Tris buffer, pH 8.2, placed on the microscope stage and an area rich in fluorescent labelling was selected. Using the capability of a confocal laser scanning microscope equipped with an AOTF and a point scanning system, a region of interest was defined and the scan zoom set to encompass only that area. Once the buffer was replaced with the DAB solution, the ROI was exposed to the appropriate wavelength laser until fluorescence was photobleached and a substantial brown reaction product was formed, as assessed by brightfield imaging. Specifically, on the Leica confocal laser scanning microscopy, the AF568 fluorophore was photoconverted with the AOTF set to transmit 100% of the 594 nm HeNe laser power and the DAPI marker was photoconverted with the AOTF set to transmit 30% of the 405nm diode laser power. The Alexa Fluor 488 (AF488) labeled BrdU-positive cells were photoconverted on the Zeiss confocal laser scanning microscope with the AOTF set to transmit 100% of the power of the 488 nm line of the Ar laser. Alternatively, a confocal system not equipped with an AOTF can still achieve photoconversion of a specific target by increasing the scan zoom alone so that it encompasses only that target, though photoconversion of an irregularly shaped ROI cannot be accomplished.

It is important to note that the photoconversion procedure was carried out without coverslipping the reacting sections, regardless of fluorophore or microscope used. This allowed the sections free access to oxygen during the reaction, a requirement necessary for successful photoconversion (Maranto, 1982; Sandell and Masland, 1988; Deerinck, 1994).

Transmission Electron Microscopy

After photoconversion, selected sections were examined under a dissecting microscope; the region containing photoconverted cells (approximately 2 × 2 mm) was dissected and then processed for TEM. Briefly, tissue was post-fixed in 1% OsO₄ for 1 hour, dehydrated through a graded ethanol series and infiltrated overnight in epon/araldite. The samples were

then flat embedded in epon/araldite and polymerized at 60° C overnight. Thin sections (< 80 nm) were collected onto formvar coated grids, stained with uranyl acetate and lead citrate and observed with a JOEL JEM-1230 transmission electron microscopy at 100 Kv.

Oligodendrocyte/Dorsal Root Ganglion (DRG) Co-culture

Oligodendrocytes and DRGs were isolated from P2 and E16 rats, respectively, and co-cultured as previously described (McCarthy and DeVellis, 1980; Sharma and Bigbee, 1998). DRG processes were immunolabeled using a mouse primary antibody to the neuronal marker, mono β -tubulin TuJ1 (1:500; Covance, MMS-435P), and Alexa Fluor 488 (1:500; Invitrogen, A11017) goat anti-mouse IgG as previously described (Sharma and Bigbee, 1998). The photoconversion of DRG processes with the 40 $\times$ (0.55 N.A.) objective lens using conventional widefield microscopy and their subsequent visualization with TEM is as described above.

Results

To establish a baseline for the procedural duration and selectivity of conventional microscopy-based photoconversion, coronal sections of a postnatal day 14 (P14) Sprague-Dawley rat cerebral hemisphere were fluorescently immunolabeled with a glial fibrillary acidic protein (GFAP) primary antibody conjugated to an Alexa Fluor 568 secondary antibody. Photoconversion was achieved with an Olympus IX70 widefield microscope using both 20 $\times$ (0.4 numerical aperture (N.A.)) and 40 $\times$ (0.55 N.A.) objective lenses. In both instances, illuminating sections with fluorescent light in the presence of a DAB solution resulted in a circular field of photoconverted astrocytes that matched the area exposed by the objective lens (fig. 1a-c). Moreover, photoconversion times varied with objective such that it required 55 minutes for the 20 $\times$ objective lens and 35 minutes for the 40 $\times$ objective lens (fig. 1 graph).

In contrast to photoconversion times achieved by conventional widefield microscopy, a dramatic decline in the photoconversion time of Alexa Fluor 568 immunolabeled sections was observed for each of the objective lenses tested on a Leica TCS-SP2 (AOBS) inverted confocal laser scanning microscope. Specifically, it took an average of 20 minutes using the 20 $\times$ (0.7 N.A.) objective lens, 10 minutes using the 40 $\times$ (1.25 N.A.) objective lens, and 7 minutes using the 63 $\times$ (1.4 N.A.) objective lens (fig. 1 graph). Interestingly, an even more dramatic reduction in photoconversion time was achieved when a 10 $\times$ (0.33 N.A.) objective lens mounted on a Zeiss LSM 510 META NLO upright confocal laser scanning microscope was used to illuminate Alexa Fluor 488 (Invitrogen) labeled BrdU-positive cells in the presence of DAB. The high-intensity lasers, elevated numerical aperture objective lenses and decreased area of scanning (i.e. increased scan zoom and small regions of interest (ROI)) offered by confocal laser scanning microscopy increased the amount of incident light per unit area and played a large part in reducing reaction times. Collectively, the results obtained using conventional widefield microscopy-based photoconversion were in agreement with previous studies (Lubke, 1993; Singleton and Casagrande, 1996; Kazca et al., 1997), whereas those obtained using confocal laser scanning microscopy-based photoconversion demonstrated that this approach could dramatically reduce photoconversion reaction times. Based on the history of successful photoconversion of various fluorophores (Lubke, 1993), it is likely this trend will translate well to numerous fluorescent labels.

Since a galvanometer driven laser scanning confocal microscope equipped with a galvanometer driven point scanning system and an acousto-optical tunable filter (AOTF) offers scan zoom and region of interest (ROI) scanning (i.e. regionally defined beam blanking) as part of its imaging capabilities, we next sought to determine the extent to which

these features affect the targeting specificity of the photoconversion procedure. Specifically, ROI scanning was used to circumscribe a single astrocyte in a population of astrocytes fluorescently immunolabeled with Alexa Fluor 568 for photoconversion (fig. 1d-f). Once the cell had been circumscribed, the scan zoom was increased to encompass only the area defined by the ROI. This typically resulted in a field of view (FOV) ranging from 100 to 400 μm^2 . The AOTF was then configured such that the 595nm HeNe laser was selected to illuminate the ROI at 100% of output power (and 0% for areas outside the defined ROI); all other laser lines were set to 0% transmission. Upon immersion in the DAB solution and laser illumination of the target, examination of the area revealed a complete loss of fluorescence and concurrent manifestation of the reaction product exclusively in the astrocyte targeted (fig 1d-f). This shows that the ROI scanning capabilities of confocal laser scanning microscopy can be used to target a single fluorescent cell amongst a population, thus enhancing the selectivity of the photoconversion procedure and representing a novel improvement over the selectivity of conventional microscopy-based photoconversion.

Utilizing a similar approach, we next tested whether the ROI scanning capability could be used to selectively target one fluorophore of a double-immunolabeled section (fig. 2a-c). Specifically, an ROI was defined for a selected astrocytic nucleus in a section fluorescently immunolabeled with AF568 and DAPI. The AOTF was adjusted such that 405 nm diode laser was set to illuminate the nucleus at 30% of its total output power. All other laser lines for the ROI and background areas were set to 0% transmission, ensuring only the nucleus would be photoconverted while sparing the astrocytic processes. Upon immersion in the DAB solution and photobleaching the fluorescent signal, only the targeted nucleus was photoconverted (fig. 2a-c). Using the same methodology, we sought to photoconvert a selection of newly generated cells in an adult rat hippocampal section immunolabeled for both BrdU and synaptophysin, and examine their appearance at the EM level (fig. 2d-f). Using ROI targeting, several BrdU-positive cells fluorescently tagged with Alexa Fluor 488 were photoconverted and could be easily distinguished with brightfield imaging from background cells by the presence of electron-dense DAB reaction product (fig. 2e). The presence of reaction product was confirmed by examining the photoconverted cells using transmission electron microscopy (fig. 2f). Taken together, these experiments demonstrate the capability of confocal laser scanning microscopy to selectively photoconvert a double-immunolabeled cell or section in a fluorophore-specific manner.

We next sought to exploit the unique features of ROI scanning to develop novel methods that would facilitate the localization and identification of photoconverted cells. Contrary to conventional widefield fluorescence microscopy, photoconverted areas generated with confocal microscopy can be tailored, allowing for varying sizes and shapes of electron dense reaction products. This flexibility allows for the enumeration of cells within a fluorescent field and facilitates the generation of directional cues for EM localization (fig. 2g-j, l). Specifically, adjacent fluorescent cells can be distinguished from one another for ultrastructural studies by simultaneously photoconverting one or more 1 μm “tags” within the cell’s cytoplasm (fig. 2g-j). More importantly, by exposing only a small area of the cell to photoconversion, ultrastructural damage common to conventional microscopy-based photoconversion is greatly reduced (fig. 2j, k). Moreover, symbols or shapes of varying sizes can be photoconverted in a fluorescent field so as to aid in the localization of cells of interest at the EM level (fig. 2l). This versatility offered by confocal laser scanning microscopy further points to its advantage over conventional microscopy-based photoconversion.

Discussion

In this study we compared and contrasted conventional widefield fluorescence and confocal laser scanning microscopy-based photoconversion so as to determine whether the

technologically distinct capabilities of confocal laser scanning microscopy can facilitate improvements to this method. In agreement with other studies, we demonstrated that photoconversion reaction times were inversely correlated to objective lens magnification and numerical aperture for both the conventional widefield and confocal laser scanning microscope. Moreover, for confocal laser scanning microscopy, the rate of photoconversion is inversely related to the degree of scan zoom and ROI size, reflecting the photon dose per unit area. For all magnifications, the photoconversion times were dramatically reduced when using the confocal laser scanning microscope in the photoconversion process, which is likely the result of the higher energies delivered by the lasers to the targets.

Additionally, we demonstrated that the ROI scanning capabilities of a confocal laser scanning microscope equipped with an AOTF represented a unique tool to facilitate the targeting of the photoconversion process to individual cellular or subcellular elements within a fluorescent field. This specificity was shown to be far superior to conventional widefield microscopy-based photoconversion where target selectivity directly mirrors objective aperture size. Moreover, photoconverting with ROI scanning provides a unique method to discretely label fluorescent cells with an electron dense tag. This method creates a means to enumerate adjacent cells of interest so they can be clearly distinguished at the ultrastructural level. It also greatly reduces the area of the cell exposed to light energy, ameliorating the ultrastructural damage common to this process when widefield illumination is employed. Furthermore, directional cues can be created to enable easy localization of cellular or subcellular photoconverted elements during EM preparation and analysis. Collectively, these experiments demonstrate that confocal laser scanning microscopy-based photoconversion facilitates a reduction in reaction time while increasing target selectivity, thereby representing a major advancement over conventional widefield microscopic methods of photoconversion.

This manuscript has not described the full spectrum of advances that can be achieved by applying confocal laser scanning microscopy to the photoconversion procedure. For example, it can be inferred that the ROI scanning technology can be used to photoconvert multiple cells simultaneously, in less time than conventional microscopy. Moreover, it is likely that the ROI scanning technology can facilitate concurrent photoconversion of two distinct fluorophores for simultaneous examination with electron microscopy. Further advances in confocal microscopy-based photoconversion will likely be uncovered as this methodology gains more widespread use. Additionally, the applications for the new methodology are not restricted to the neurosciences. Certainly, this method can be employed by any scientific modality that requires the ultrastructural analysis of fluorescently labeled cellular elements, especially those where photoconversion in a highly targeted manner could be beneficial.

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Figure 1. Comparison of cell selectivity and photoconversion time between conventional widefield and confocal laser scanning microscopy

(a) P14 rat astrocytes immunolabeled for GFAP, viewed with widefield fluorescence microscopy and a 20x objective lens before photoconversion. The circle corresponds to the region selected to be photoconverted. (b) Area shown in a, viewed after photoconversion. The dark circle corresponds to the field photobleached by fluorescent light using the 40x objective lens. (c) Bright field microscopy of area shown in a, after photoconversion. Note the photobleached area in b corresponds to the area in c where previously fluorescent astrocytes now contain a brown reaction product. (d) P14 rat astrocytes immunolabeled for GFAP, viewed with confocal laser scanning microscopy before photoconversion. The square denotes an approximation of the ROI selected for photoconversion and corresponds to the squares in e-g. (e) Astrocytes shown after photoconversion using the 594 nm HeNe laser at 100% power. Note the fluorescence is bleached only from the region of interest selected. (f) Differential interference contrast (DIC) image shown taken after photoconversion. Note the appearance of reaction product in the area selected. The astrocytic processes are readily visible. (g) Chart comparing the photoconversion time of AF568 using the Olympus widefield microscope and the Leica CLSM. Scale bars: a-c = 0.5mm; d-g = 20 μ m.

Fig 2. Increased selectivity exhibited by confocal laser scanning microscopy

(a) Fluorescent image of P14 rat brain with astrocytes immunolabeled for GFAP (red) and cell nuclei labeled with DAPI (blue, arrow) before photoconversion. (b) The same image shown after photoconverting only the nucleus (arrow) using the 405 nm diode laser set at 30% power. Using this wavelength coupled with the ROI scanning to selectively photoconvert the nucleus spares the astrocytic processes from the reaction. (c) DIC image of the same area showing the reaction product deposited after photoconversion (arrow). (d) Fluorescent image of adult rat hippocampal dentate gyrus stained for synaptophysin (red) and BrdU (green). (e) BrdU-positive cells after photoconversion with the 488 nm line of the Ar laser set at 100%. White arrow corresponds to the same BrdU cell in both a and b. (f) Transmission electron micrograph of a photoconverted BrdU-positive cell showing DAB reaction product (white arrows). (g) Fluorescent image of P14 rat astrocytes immunolabeled for GFAP before photoconversion. (h) The same image after cells were “tagged” using the 594 nm HeNe laser set at 100% power (arrows). All three “tags” were created simultaneously. (i) DIC image showing the DAB reaction product localized to the ROI selected (arrows). (j) Transmission electron micrograph showing an example of a cell that has been tagged with a small 1 μ m dot (arrow). (k) Transmission electron micrograph of a

oligodendrocyte/dorsal root ganglion cell co-culture where the DRG process (arrow) was photoconverted with conventional microscopy. Note the cytoarchitectural damage of the DRG process as compared to the preserved cytoarchitecture of the oligodendrocytes, which was not photconverted. **(I)** Brightfield image taken at 10x showing a photoconverted symbol used to demarcate small regions of interest for easy locating during EM workup and examination at the ultrastructural level. Scale bars: a-c = 5 μ m; d, e = 200 μ m; f = 1 μ m; g-i = 5 μ m; j = 2 μ m; k = 5 μ m; l = 100 μ m.