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Confocal laser scanning microscopic photoconversion: a new method to stabilize fluorescently labeled cellular elements for electron microscopic analysis

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Abstract

Photoconversion, the method by which a fluorescent dye is transformed into a stable, osmophilic product that can be visualized by electron microscopy, is the most widely used method to enable the ultrastructural analysis of fluorescently-labeled cellular structures. Nevertheless, the conventional method of photoconversion using widefield fluorescence microscopy requires long reaction times and results in low resolution cell targeting. Accordingly, we have developed a photoconversion method that ameliorates these limitations by adapting confocal laser scanning microscopy to the procedure. We have found that this method greatly reduces photoconversion times as compared to conventional wide field microscopy. Moreover, region of interest scanning capabilities of a confocal microscope facilitate the targeting of the photoconversion process to individual cellular or subcellular elements within a fluorescent field. This reduces the area of the cell exposed to light energy, thereby reducing the ultrastructural damage common to this process when widefield microscopes are employed.

Keywords

Photo-oxidation; Diaminobenzidine; ultrastructure; electron microscopy; immunolabelling; confocal microscopy

Unit Introduction

Labeling cells and tissues with fluorescent markers is one of the most widely used methods of cellular examination employed today. Fluorescence immunolabeling is a highly specific method that is commonly used in research science to facilitate cell structure visualization, protein localization, and cell interaction studies at the light microscopic level. Various fluorophores that can target a diverse assortment of cellular elements are widely available on the commercial market, making the use of these labels very easy. Unfortunately, long term study of fluorescently labeled elements is impossible because the fluorescent signal is subject to photobleaching upon illumination with light. Furthermore, fluorescent dyes are inherently undetectable by electron microscopy, making ultrastructural analysis, often a desired step in cellular examination, unfeasible.

A method has been developed, however, that enables translation of the fluorescent signal to a marker that is both permanent and compatible with light and transmission electron microscopy. This method, termed photoconversion, is the most frequently used technique to

facilitate ultrastructural examination of fluorescently labeled elements. It is accomplished by illuminating a fluorescent dye with its excitation wavelength in the presence of the chromagen, 3,3' diaminobenzidine, in solution. Under these conditions, rather than generating photons of emission (as would typically occur when excited fluorophore electrons return to the ground state, in the absence of the chromagen), intersystem crossing occurs in the presence of O₂, ultimately forming reactive singlet oxygen that will oxidize the chromagen (DAB) to form a stable, brown, electron-dense reaction product. 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.

With the advent of confocal laser scanning microscopy (CLSM), significant improvements to image contrast and spatial resolution have been achieved over conventional widefield microscopy. Specifically, confocal laser scanning microscopy offers significant improvements over conventional microscopy by utilizing high-intensity coherent monochromatic lasers to excite fluorophores more efficiently, thereby reducing the time necessary for conversion. Moreover, conventional laser scanning microscopy offers scan zoom and (if equipped with an acousto-optical tunable filter (AOTF)) specific region of interest (ROI) scanning as part of its imaging capabilities, making cellular targeting possible. This unit will briefly review the procedure of photoconversion using conventional widefield microscopy and will delineate the steps for adapting this procedure to confocal laser scanning microscopy. The commentary will describe and provide examples for how the distinct capabilities of confocal laser scanning microscopy can facilitate improvements to this method.

CAUTION

3,3'Diaminobenzidine (DAB) is a hazardous chemical and may act as a carcinogen: wear protective gear, use in a safety hood and avoid spilling. DAB is moisture and light sensitive and is incompatible with strong oxidizing agents. DAB solution can be neutralized with a potassium permanganate-sulfuric acid mixture for disposal (see Protocol Steps and Annotations). Also, use glutaraldehyde and paraformaldehyde in the hood.

NOTE

The two major safety concerns associated with the use of a confocal laser scanning microscope are exposure to the beam and the electrical hazards associated with high voltages within the laser power supply. All precautions must be taken in advance to minimize the risk associated with laser beam and high voltage exposure.

BASIC PROTOCOL 1

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 dyes have been photoconverted successfully, the fluorophores Alexa-Fluor 568 (AF568) and Alexa-Fluor 488 will be used in this unit because their excitation wavelengths are in the red and green bands of the light spectrum, away from the yellow band where glutaraldehyde-fixed tissue typically autofluoresces. 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).

In this manuscript, rat brain sections are used as a model/platform for presenting the specific steps for the photoconversion method. Therefore, steps for surgery and tissue and slide preparation are given in rough detail, with references to other protocols which explain the procedures in greater detail.

Basic Protocol Materials List

Basic Materials

Postnatal day 14 (P14) Sprague Dawley rat pups
 Pentobarbital anaesthetic
 Basic dissection kit (forceps, scissors, scalpel, microdissection scissors)
 0.17 mm (#1.5) glass coverslips, coated with poly-L-lysine
 Superfrost (Sigma) glass microscope slides
 Tissue-Tek
 PAP pen

Solutions and Reagents

poly-L-lysine
 Rabbit anti-GFAP (1:500, DAKO Z0334)
 Goat anti-rabbit IgG (1:200, Invitrogen A-11036)
 4',6-diamidino-2-phenylindole (DAPI)
 3,3'-diaminobenzidine (DAB)
 0.1M Tris buffer, pH 8.2
 4% paraformaldehyde
 0.1% glutaraldehyde
 Alexa Fluor 568 (AF568)
 isopentane
 1% Triton X-100,
 1% bovine serum albumin (BSA)
 5% normal horse serum (NHS)
 0.1M phosphate-buffered saline (PBS)

Special equipment

Olympus IX70 conventional microscope with a 100W mercury lamp and 20x (0.4 N.A.) and 40x (0.55 N.A.) objective lenses
 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), reflected & transmitted light PMTs and several objective lenses including: 20x (0.7 N.A.) dry, 40x (1.25 N.A.) oil, and 63x (1.4 N.A.) oil.

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, a Spectra-Physics Mai-Tai Ti:sapphire laser, reflected & transmitted light PMTs and several objective lenses including a 10x (0.3 N.A.) dry lens used in this protocol.

NOTE: 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).

Leica cryostat with tungsten knife

Basic Protocol Steps and Annotations

Preparation of brain sections

1. Anesthetize postnatal day 14 (P14) Sprague-Dawley rat pups and transcardially perfuse 0.1M phosphate-buffered saline (PBS) to flush blood.
2. Dissect the brain, coat with Tissue-Tek, and freeze in dry-ice chilled isopentane.
For more detailed information about tissue freezing methods, please refer to Basic Protocol 6 in Mark et al. (2007).
3. Use the cryostat to cut coronal sections at a thickness of 10 μ m.
IMPORTANT VARIATION: For conventional microscopy collect tissue sections on Superfrost glass slides, but for confocal microscopy collect tissue sections on poly-L-lysine treated glass coverslips.

Fixation

4. Treat tissue sections with fixation solution for 30 minutes (4% paraformaldehyde in 0.1M PBS; for transmission electron microscopy (TEM) add .1% glutaraldehyde to this solution).
Sections collected on slides can be circumscribed with a PAP pen to localize solutions during fixation and labeling.
5. Rinse sections in 0.1M PBS three times for 5 minutes each.

Immunofluorescent labeling of astrocytes—Although the directions of this unit apply to the immunofluorescent labeling and photoconversion of astrocytes, research has shown virtually all immunofluorescent labels to be susceptible to the photoconversion reaction. Therefore, we expect this technique to be applicable, for both conventional and confocal microscopy, to the tissue and label of your choosing (see figure 1g–i). See “Critical Parameters” for assistance on fluorophore selection.

6. Treat tissue sections with blocking solution for 30 minutes.
7. Treat tissue sections with rabbit anti-GFAP (1:500, DAKO Z0334) in 0.1M PBS with 5% NHS for one hour at room temperature.
8. Rinse sections in 0.1M PBS three times for 5 minutes each.
9. Treat sections with Alexa Fluor 568 (AF568) conjugated goat anti-rabbit IgG (1:200, Invitrogen A-11036) in 0.1M PBS with 5% NHS for one hour at room temperature.
10. Rinse sections in 0.1M PBS three times for 5 minutes each.

Some sections can be additionally treated 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 using conventional microscopy—The following steps were adapted from Maranto (1982) and Sandell and Masland (1988) and published in Tozer et al., (2007). Please refer to these key references if more detailed instructions are required.

11. Pre-treat an immunolabeled section in cold 0.1M Tris buffer, in the dark, for 5 minutes.
12. Place a slide containing an immunolabeled section in Tris buffer on the microscope stage and select area of interest for photoconversion.
13. Remove the Tris buffer using a micropipette and replace it with cold DAB solution.

Subjectively, fresher DAB solution tends to yield quicker reaction times and more dense reaction product. Therefore, the best results can be achieved using DAB solution prepared and cooled just prior to treating the immunolabeled sections.

14. Illuminate the region of interest with the appropriate wavelength of fluorescent light until fluorescence has diminished and a substantial brown reaction product had formed. As the fluorescence fades, switch the microscope to brightfield in order to assess the reaction product formation.

See figure 1a, b, and c.

15. When the desired level of reaction product has formed, remove the DAB solution from the tissue sample using a micropipette and rinse the sample three times with 0.1M PBS for five minutes each.

Because the reaction product is stable, the slide can be stored at room temperature until such time as you are ready to prepare the sample for electron microscopy.

Photoconversion using confocal laser scanning microscopy

Follow steps 1–10 for tissue collection, fixation, and immunolabeling. Then follow steps 11 and 12 for tissue preparation prior to the photoconversion procedure. Remember to collect tissue sections to be examined with confocal microscopy on glass coverslips. Because an inverted confocal laser scanning microscope is used, this reduces light scatter through the glass and accommodates the working distances of the high N.A. objective lenses used, thereby minimizing spherical aberrations.

NOTE: The operation of a confocal laser scanning microscope is a science unto itself. Its use requires significant training and practice to become proficient. We recommend consulting an expert if you are unfamiliar with the operation of these devices. See the section titled 'Special Equipment' for the exact parameters of the microscopes used.

16. Using the capability of a CLSM equipped with an AOTF and a point scanning system, select area to define as the region of interest (ROI) and set the scan zoom to encompass only that area.

The ROI capability of a CLSM enables the user to carry out the photoconversion reaction on only the area of their choosing. See figure 1d–

i. Furthermore, the ROI capability enables the specific photoconversion of discrete cellular elements (see figure 2).

Alternatively, a confocal system not equipped with an AOTF can still achieve photoconversion of a specific target by increasing the scan zoom so that it encompasses only that target, though photoconversion of an irregularly shaped ROI cannot be accomplished.

17. Replace the Tris buffer solution with DAB using a micropipette as in the conventional procedure.

18. Expose the ROI to the appropriate wavelength laser to achieve the photoconversion reaction.

For example, the AF568 fluorophore was photoconverted using 594 nm HeNe laser set at 100% power and the DAPI marker was photoconverted using the 405nm diode laser set at 30% power. The best results, in terms of successful photoconversion and reduction in reaction time, can be achieved using the highest N.A. lenses at your disposal (see figure 3).

19. Use differential interference contrast (DIC) imaging to assess the reaction product formation.

See figure 1f.

20. Repeat step 15 when your sample has ceased to fluoresce and a substantial reaction product has formed.

Examination of the reaction product using electron microscopy—See unit 2B.1 (Current Protocols in Microbiology, 2006) for instructions on preparation of the tissue samples for electron microscopy

Reagent and Solutions

Poly L-lysine coating of glass coverslips

Coat acid-washed coverslips with 1mg/ml Poly L lysine (MW 300k) in ddH₂O for 30 minutes. Rinse 3 times with ddH₂O and let dry before use.

Fixation solution

4% paraformaldehyde

0.1% glutaraldehyde

0.1M PBS

buffer to pH 7.4

Blocking Solution

0.1M PBS

1% Triton X-100,

1% bovine serum albumin

5% normal horse serum

Tris buffer solution

0.1M Tris

buffer to pH 8.2

DAB Solution

1.5mg/mL DAB

0.1M Tris buffer,

adjust pH to 8.2

store at 5°C for no more than 2 days

Commentary

a. Background Information

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, unstable during prolonged storage and are, therefore, poor permanent labels (Lubke, 1993). Moreover, they are inherently undetectable by transmission electron microscopy (TEM) because of their low electron density (Lubke, 1993). These shortcomings created the need for a technique that converts the signal from a 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 the signal from 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 excite fluorophores more efficiently, thereby reducing the time necessary for conversion. Moreover, conventional laser scanning microscopy offers scan zoom and (if equipped with an AOFT) specific region of interest (ROI) scanning as part of its imaging capabilities, making cellular and subcellular targeting possible. Consequently, replacing conventional widefield fluorescence microscopy with confocal laser scanning microscopy in the photoconversion process enables the selective targeting of fluorescently labeled structures while concurrently reducing the overall reaction time (Tozer et al., 2007).

b. Critical Parameters

There are a number of points to consider, both in terms of sample preparation and hardware used, for this procedure to work optimally. The labeling of the tissue sample must be specific, with a reasonably high signal / noise ratio. If immunolabeling is used, both the primary and secondary antibodies must have a high degree of localization specificity with a low background. The process of photoconversion is based the presence of a detectable

amount of fluorophore, and will occur wherever the fluor resides. Thus, if there is a high degree of background or spurious localization of the antibodies, reaction product will be formed in inappropriate locations. Additionally, the signal intensity of the labeling should be much greater than the background. Weak signals will require prolonged exposure to the laser for sufficient photoconversion to have a detectable reaction product. If the exposure time is too long, there will be a greater degree of ultrastructural damage due to the formation of free radicals as well as a higher rate of photoconversion of the background regions.

Although we have yet to make a systematic study of the efficiency of photoconversion of various fluors available, the fluor used should excite easily and be relatively bright. Fluors with reasonably high quantum yields and high extinction co-efficients should be used. To date, we have used this procedure successfully with DAPI, FITC, Alexa488 and Alexa568. If there is an abundance of autofluorescence in the tissue to be studied, a fluor should be selected that has an excitation peak that is outside of the excitation range of the autofluorescence. For samples that are labeled with 2 (or more) colors, fluors should be selected with distinct excitation spectra. As each fluor may be excited over a range of wavelengths (with a maximum excitation frequency at a defined wavelength), it should be insured that the laser line used to excite one fluor optimally will not also excite another fluor in the same area with a lesser degree of efficiency. If two different fluors that may be excited with a common laser line are used, following photoconversion, there would be no way to distinguish the reaction product generated by each. Thus, two different fluors that both may be excited (to with varying efficiencies) with the same laser line, should not be used for double labeling.

For excitation, laser lines used for photoconversion should be chosen to match, as close as possible, the maximum excitation frequency for the fluor used. Conversely, fluors used for photoconversion should be selected so as to obtain the maximum excitation efficiency from the available laser lines of the confocal microscope. As an example, although a 543 nm HeNe laser may be used to excite Alexa568, a 561 nm diode pumped solid state laser will provide greater efficiency in exciting the fluor and, thus, shorter exposure times, resulting in less photodamage of the sample and less background photoconversion.

The configuration of the microscope stand has some influence on the efficiency of photoconversion in terms of which objective lenses may be used and how the sections are mounted. Whether performed on an upright or an inverted microscope stand, the degree of scatter of the illuminating light needs to be minimized. As such, if an inverted microscope stand is used, tissue sections should be collected on a coverslip rather than a microscope slide. By supporting the section on the thinner coverslip (vs. the thicker glass) the amount of scatter of the illuminating light is minimized, thus increasing the efficiency of photoconversion. The collection of sections on a coverslip also permits the use of high numerical aperture (NA) immersion lenses when used on an inverted microscope stand. The NA of the objective lens used has a significant impact on the effectiveness of the photoconversion process. The higher the NA of the objective time used, the greater the efficiency of photoconversion and the shorter the exposure time required to generate a reaction product (figure 3).

If an upright microscope stand is used, the substrate onto which the tissue section is collected may be either a slide or a coverslip. However, as the DAB and the objective lens will both be on the same side of the substrate (unlike the configuration on an inverted stand, where they are on opposite sides of the substrate) immersion lenses (with higher NA) may not be used. Most immersion lenses require a coverslip between the lens and the tissue section. For the purposes of photoconversion with an upright microscope stand, the overlaying of the tissue slice (and DAB) with a coverslip is undesirable, as this reduces the

amount of exposure of the DAB to environmental oxygen. However, even with lower NA lenses, photoconversion on an upright microscope stand can be very efficient, given sufficient laser power and scan zoom. In some cases, working with an upright configuration has produced even faster rates of photoconversion, likely due to the absence of the light-scattering coverslip between the laser and the DAB (as occurs when working with an inverted microscope stand).

To produce the shortest photoconversion times, the scan zoom of the confocal microscope should be set as high as is needed (i.e. the smallest area possible) to encompass the desired feature to be photoconverted. The higher the scan zoom, the shorter the photoconversion time, as the photon-density is increased over the smaller area covered by the illuminating laser. Additionally, by zooming in tight on the feature of interest, the possibility of photoconverting adjacent regions that are not of interest is minimized. If the confocal microscope is equipped with an AOTF, it is also possible to generate region of interest (ROI) scans, by blanking the laser in regions outside of the selected area of interest. By combining ROI scanning with scan zoom, specific irregularly shaped areas may be photoconverted in brief periods of time. Furthermore, ROI scanning permits the possibility of ‘tagging’ different cells with distinct shapes, thus providing the potential to examine multiple cell types or multiple signals detected with different fluors. This flexibility allows for the enumeration of cells within a fluorescent field and facilitates the generation of directional cues for EM localization. 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 (figures 2d–f). Furthermore, to eliminate the possibility of any ultrastructural photodamage to a labeled cell, ROI scanning may be used to ‘tag’ one or more fluorescently labeled areas surrounding the cell of interest, thus isolating the cell for ultrastructural analysis without actually photoconverting the fluorescence within that cell. This versatility offered by confocal laser scanning microscopy further points to its advantage over conventional microscopy-based photoconversion.

c. Troubleshooting

As mentioned above, it is desirable to minimize the duration of exposure of the tissue sample to the laser so as to minimize the degree of phototoxic damage to the ultrastructure as well as minimize the amount of non-specific signal from the photoconversion of background fluorescence. If the times for photoconversion are too long, the intensity of the illumination source may be increased. This may be accomplished in several different ways. If the confocal system uses an AOTF to regulate the illumination source, the AOTF may have a default setting that is less than 100% transmission of the laser line. If this is the case, the setting may be increased to maximum. Also, a higher NA objective lens may be used. The scan zoom should be maximized such that the scan range covers only the area of interest, thus maximizing the photon density per unit area. If the confocal scan is set by default to blank on the return stroke during the scan, this may be turned off such that the sample is exposed to the laser during both the forward and return sweep of the laser.

If multiple laser lines are available, the line that provides the greatest efficiency of excitation of the fluor should always be used. If the laser line used is too far removed from the excitation maximum peak for a fluor (thus exciting the fluor inefficiently) and no other suitable laser line is available, another fluor should be used that is more compatible with the available illumination source.

If there is bright fluorescent signal, with a high signal:noise ratio and a low degree of autofluorescence of the tissue, the process of photoconversion should be straightforward. If there is a high degree of tissue autofluorescence that is excited with the laser line that is used to illuminate the reporter fluor, there may be a high degree of non-specific confounding

reaction product. In such cases, another reporter fluor should be chosen that excites with a laser line that is removed from and does not excite the tissue autofluorescence. Furthermore, as stated above, if tissue sample is labeled with two different fluors that are each excited, with varying degrees of efficiency, by the same laser line, there is a possibility of undesired reaction product due to cross-excitation of fluors. For double labeling, fluors should be chosen with spectrally distinct excitation wavelengths and minimal excitation efficiency when illuminated with the laser line used to excite the other fluor.

In our experience with this technique, the most troublesome issue has been the quality of the DAB used for photoconversion. The DAB should be recently purchased and the solution prepared immediately prior to the photoconversion. Older batches of DAB provide poor results. Often we have purchased batches of DAB that do not appear to work at all well in our hands. In these cases, we have rejected these batches and purchased new batches of a different lot number from the vendor.

d. Anticipated results

If the conditions outlined above are fulfilled, the process of photoconversion should occur within a few minutes. During this time, as the laser is scanned over the region of interest, a progressive fading of the fluorescent signal in the reflected light channel of the confocal microscope will be noted. At the same time, if the microscope is equipped with a transmitted light detector, it will be possible to monitor the accumulation of the light scattering (i.e. opaque) reaction product of the photoconversion. The outline of this reaction product should closely match the original pattern of fluorescence prior to photoconversion.

e. Time Considerations

There are a number of factors that will influence the duration of the photoconversion process. As noted above, the NA of the objective lens should be as high as is practicable for the configuration of the microscope used. The amount of time for photoconversion will decrease with a higher NA (see figure 3).

The degree of light scattering will also affect the rate of photoconversion. If the illumination source must pass through glass and/or a thick tissue slice to reach the fluorescent target, the increase in light scatter will reduce the rate (i.e. increase the time) of photoconversion. If illuminating from below (i.e. with an inverted microscope stand) the use of a coverslip as a support will reduce the time for photoconversion. If illuminated from above, the amount of light scatter is reduced due to the lack of intervening coverslip. In either configuration, a thinner tissue section will photoconvert more efficiently than a thicker slice.

The power of the laser as well as the AOTF setting will influence the rate of photoconversion. Low power lasers and/or low AOTF settings will increase photoconversion times.

Under the conditions described herein using a confocal laser scanning microscope, the process of photoconversion should occur within a few minutes. Depending upon the lens used, the microscope configuration and the laser power, the duration for photoconversion can require from 1–3 minutes up to 30 minutes.

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Figure 1. Comparison of cell selectivity 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 figure 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 figure 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) Fluorescent image of adult rat hippocampal dentate gyrus stained for synaptophysin and BrdU. (h) 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 g and h. (i) Transmission electron micrograph of a photoconverted BrdU-positive cell showing DAB reaction product (white arrows). Scale bars: a-c = 0.5mm; d-g = 20µm.

Figure 2. Increased selectivity exhibited by confocal laser scanning microscopy

(a) Fluorescent image of P14 rat brain with astrocytes immunolabeled for GFAP and cell nuclei labeled with DAPI (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 P14 rat astrocytes immunolabeled for GFAP before photoconversion. (e) The same image after cells were “tagged” using the 594 nm HeNe laser set at 100% power (arrows). All three “tags” were created simultaneously. (f) DIC image showing the DAB reaction product localized to the ROI selected (arrows). Scale bars: a–c = 5µm; d, e, f = 1µm.

Figure 3. Photoconversion times

Chart comparing the photoconversion time of AF568 using the Olympus widefield microscope, the Leica CLSM and the Zeiss two-photon confocal microscope.