A New Low Cost Wide-Field Illumination Method for Photooxidation of Intracellular Fluorescent Markers

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Abstract

Analyzing cell morphology is crucial in the fields of cell biology and neuroscience. One of the main methods for evaluating cell morphology is by using intracellular fluorescent markers, including various commercially available dyes and genetically encoded fluorescent proteins. These markers can be used as free radical sources in photooxidation reactions, which in the presence of dianaminobenzidine (DAB) forms an opaque and electron-dense precipitate that remains localized within the cellular and organelle membranes. This method confers many methodological advantages for the investigator, including absence of photo-bleaching, high visual contrast and the possibility of correlating optical imaging with electron microscopy. However, current photooxidation techniques require the continuous use of fluorescent or confocal microscopes, which wastes valuable mercury lamp lifetime and limits the conversion process to a few cells at a time. We developed a low cost optical apparatus for performing photooxidation reactions and propose a new procedure that solves these methodological restrictions. Our “photooxidizer” consists of a high power light emitting diode (LED) associated with a custom aluminum and acrylic case and a microchip-controlled current source. We demonstrate the efficacy of our method by converting intracellular Dil in samples of developing rat neocortex and post-mortem human retina. Dil crystals were inserted in the tissue and allowed to diffuse for 20 days. The samples were then processed with the new photooxidation technique and analyzed under optical microscopy. The results show that our protocols can unveil the fine morphology of neurons in detail. Cellular structures such as axons, dendrites and spine-like appendages were well defined. In addition to its low cost, simplicity and reliability, our method precludes the use of microscope lamps for photooxidation and allows the processing of many labeled cells simultaneously in relatively large tissue samples with high efficacy.

Introduction

Analyzing cell morphology is a crucial aspect of cell biology and neuroscience. Relationships between form and function define physiological processes in health and disease. One of the main methods for evaluating cell morphology is through the use of intracellular fluorescent markers, including various commercially available dyes, fluorochrome labeled antibodies and genetically encoded fluorescent proteins, such as green fluorescent protein (GFP). These fluorophores can be visualized directly under fluorescent, confocal or multi-photon microscopy, allowing the investigator to directly observe the targeted cell, including cellular substructures. Fluorescent markers can also be used for conventional bright field optical microscopy and transmission electron microscopy through a process called fluorescent photooxidation. This technique, initially developed by Maranto [1], is based on photoconverting certain intracellular markers in order to promote the oxidation of 3,3’-dianaminobenzidine tetrahydrochloride (DAB) with high spatial precision and acuity. This is possible due to the fact that most organic fluorophores release singlet oxygen molecules when illuminated. In the presence of a DAB solution, the reactive free radicals oxidize this organic compound, forming an opaque, electron-dense and osmiophilic brown polymer [1,2]. Because singlet oxygen molecules are only released where there are fluorescent markers and exclusively upon illumination with an adequate light wavelength, the investigator has sufficient control of the temporal and spatial parameters of the photooxidation reaction, thus being capable of regulating the structural specificity and intensity of the subsequent DAB staining. In addition, the resulting staining does not suffer from photobleaching and can be applied to correlative light and transmission electron microscopy [1,2].

While photooxidation was initially executed with Lucifer Yellow, it has been subsequently demonstrated in a variety of cell types that other fluorescent dyes can be applied for the same purpose, provided they are exposed to light with a spectrum that corresponds to their excitation wavelength [2]. Examples of fluorophores that have been used for fluorescent photooxidation include DiI, DiO, Fast Blue, Fluorogold and rhodamine labeled microspheres [3–8]. These dyes have the advantage of providing...
A Novel Method for Photooxidation

Materials and Methods

Ethics statement

All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), and were approved by the Ethical Research Committee for Animal experiments of the Institute of Biological Sciences, Federal University of Paraíba (protocol N°: 3989/2011). Animals were obtained from the Animal Service of the Institute of Biological Sciences of the Federal University of Paraíba. Samples of fixed post-mortem human retinas were obtained from the Optir Loyola Hospital’s Eye Bank (Belém, PA, Brazil). Written statements from family members of the deceased subjects is a prerequisite for tissue storage in this bank. All procedures with post-mortem human tissue were approved by the Committee on Research Ethics of the Federal University of Paraíba (protocol N°: 2012/20468).

Photooxidizer apparatus

For our apparatus, we used a high power LED (XR7090GRN, Cree) powered by a voltage regulator microchip (LM 317, Texas Instruments). The nominal current for this LED is 350 mA for a 3.6 Ω resistor, but we chose to use an associated resistor of 3.9 Ω, resulting in a current of 320 mA to the LED. This difference does not affect the photooxidation process, but increases the LED's lifespan substantially. A red low power LED indicates if the circuit is being fed (Figure 1, Panels B-D). The complete circuit was mounted in a circular printed circuit board (Figures 1 and 2). The spectral emission of the high power LED was measured with a spectroradiometer (SpectraScan 705; Photo Research, USA) and is represented graphically in Figure 2, Panel C.

The high power LED is fixed within a cylindrical aluminum chamber. The LED inner circuit is isolated from the aluminum chamber by a Teflon® ring. A heat sink (an aluminum grooved plate) is screwed to the underside of the LED chamber and thermal paste is added to allow a more effective heat transfer. The LED chamber and the PCB are fixed within a cylindrical transparent acrylic case. The case is equipped with an external power connector (NIM-CAMAC 00.250 series - fixed socket, Lemo, Switzerland) and an on/off switch (Spot micromini toggle switch, RadioShack, USA) that controls the high power LED’s function. At the underside of the acrylic case an aluminum heat sink is added, necessary for dissipating the 1.8 W of power generated by the PCB’s circuit. For detailed information on the circuit board configuration and photooxidizer assembly see Supplemental Information (Tables S1 and S2; File S1).

Tissue chamber

During the photooxidation reaction, tissue samples are placed within a custom-made tissue chamber (Figure 1E). This piece consists of a translucent acrylic ring with a glass coverslip glued to its underside. The inner hole of the ring has a conical recess, in order to better accommodate the objective lens of a microscope. This chamber allows the investigator to transfer the samples from the photooxidizer to the microscope and back easily, thus facilitating the monitoring of the photooxidation reaction, a critical factor for obtaining high quality images. In order to avoid light dispersion, the chamber has a tightly fit translucent acrylic lid. Tissue samples are placed in a stainless steel ring with an internal nylon mesh (Figure 1E).

In order to ensure that the LED chamber heat sink was capable of successfully dissipating the heat generated by the illumination process, we filled the tissue chamber with deionized water (resistance of 18.6 MΩ) and measured the temperature variation within the chamber during four 15 minute bouts of continuous illumination, i.e. the same duration of illumination bouts used for controlled photooxidation reactions. Temperature measurements were carried out with a Fluke 51 K/J digital thermometer (Fluke Corporation, USA). We found that, starting from a mean room temperature of 25.55 °C (SDM = 0.24), continuous LED illumination caused only a mean increase of 3.52 °C (SDM = 0.17) within the chamber, with the maximum temperature not exceeding 29.3 °C. This level of temperature increase clearly does not affect tissue integrity and did not affect photooxidation quality in our experiments (Figures 3–5, Table 1 and Figure S3), confirming that the heat dissipation system is functional and adequate for the desired purposes.

Animal manipulation procedures and collection of tissue samples

Samples of developing rat neocortex were obtained from Wistar rats at days P4–P6 of postnatal development. The rats were deeply anesthetized with ketamine (12 mg/kg; Vetanarcol, Konig, Brazil) and xylazine (90 mg/kg; Rompun, Bayer, Germany) and the level of anesthesia was assessed based on the animal’s reaction to a toe pinch. The rats were then decapitated and their brains were removed and immersed in formaldehyde 4%. After at least two weeks of post-fixation, rat brains were mounted on a custom built vibratome and either tangential or coronal sections (70–100 μm thick) were obtained from area 17 of the visual cortex. Brain slices were then stored in formaldehyde 4% at 4°C until further procedures. Human retina samples were surgically removed during autopsy and stored in formaldehyde 4% at 4°C for at

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least two weeks. They did not receive any further treatment apart from dye labeling and histological processing.

Histological processing and dye diffusion

Cells were labeled with the fluorescent, lipophilic carbocyanine dye DiI (1,1',dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate; Invitrogen, USA). As shown in Figure 2C, the emission spectrum of the photooxidizer apparatus encompasses the excitation wavelength of DiI (549 nm). A hypodermic needle was used to insert small beads of DiI into the parenchyma of each sample at several representative locations. The tissue was then stored in formaldehyde 4% at room temperature (<23°C) for 1–4 weeks to allow for dye diffusion across the cellular membrane. After this period, DiI labeled cells were observed using fluorescent microscopy (Eclipse 80i microscope; Nikon, Japan) to confirm they were appropriately stained (Figures S1 and S2).

Photooxidation protocol

Fluorescent marker photooxidation was performed using a modified version of the protocol by Sandell and Masland [4]. Briefly, the tissue samples were incubated in a chilled solution of 0.15% DAB (Sigma, USA) in 0.1 M Tris/HCl buffer (pH = 8.2). The tissue was held in place by the nylon mesh ring, directly on top of the LED’s light beam. The DAB solution of the tissue chamber was changed every 15 minutes until the end of the protocol. The photooxidation process was periodically monitored using a low power light microscope (Standard 20; Zeiss, Germany) and lasted between 120–180 min. After photooxidation, tissues were washed overnight in 0.1 M Tris/HCl buffer and then mounted on a glass slide with Mowiol (Polysciences, USA).

In order to compare the efficiency of our method with currently standard illumination techniques, we performed control photooxidation experiments using a conventional broad spectrum mercury
lamp as a light source. Microscope illumination was performed through a 520–553 nm light filter (G-2E/C; Nikon, Japan) and a 10x objective lens on an Eclipse 80i microscope (Nikon, Japan) with a mercury arc fluorescence lamp as the illumination source. The photooxidation protocol and histological procedures were exactly the same as those used for reactions in the photooxidizer, with the sole exception that the monitoring of staining quality was performed directly on the Eclipse 80i microscope.

Image acquisition and comparison with conventional methods

Photooxidized tissue samples were visualized under bright field microscopy with an Eclipse 80i microscope and microphotographed at maximum resolution with a Nikon DS-Fi1c camera (Nikon, Japan). Photomicrographs were taken across various focal planes and then merged with the Helicon Focus® software (Helicon Soft, Ukraine) in order to allow the full visualization of neuronal processes across the depth axis. All images were adjusted for brightness and contrast across their entire span.

For the measurement of illumination field areas, successfully photooxidized tissue samples were photographed at very low magnifications with a M165 C stereomicroscope (Leica, Germany). The illumination field was defined as the area of the tissue sample that was selectively photooxidized during one procedure. Quantification of this area was performed using ImageJ software by defining a circle that encompassed the photooxidized cell bodies and neurites with the “elliptical selection” tool and then measuring its area with the “measure” command.

Results and Discussion

Staining quality

Our new method for fluorescent marker photooxidation yielded high quality and detailed images of neuronal morphology in nearly all processed tissue samples (Figures 3 and 4). Numerous cell bodies, dendritic arbors, dendritic appendages and axonal projections were labeled in all DiI-stained regions (Figures 3 and 4). Dendritic terminal clusters (Figure 4, Panels A–C) and axon terminal boutons (Figure 4, Panel D) could be clearly identified. Very similar results were obtained in control photooxidation reactions performed using the Eclipse 80i microscope, which confirms that the quality of photooxidation-based DAB staining performed with the new apparatus is on equal footing with current standard methodology (Table 1 and Figure S3). Likewise, the total illumination time necessary for high quality staining of neuronal cells was similar for both methods: 2 to 3 hours (Table 1). We were also capable of reliably visualizing the same set of cells under fluorescent (pre-photooxidation) and conventional (post-photooxidation) light microscopy without any obvious loss in staining quality (Figure S3).

In samples of developing rat neocortex, we retrieved images of DiI labeled Cajal-Retzius cells (Figure 3). The large unipolar horizontal-projecting dendrite of the Cajal-Retzius cell was clearly identified, as well as the dendritic side branches and spine-like appendages that are typical of this cell type (Figure 3, Panels A–B). The detailed visualization of these dendritic appendages highlights the acuity of the new method.

We were also able to successfully photooxidize DAB in fixed post-mortem human retinas. Most labeled neurons were identified as putative horizontal cells (Figure 4, Panels A–C). Photomicrographs of DAB-labeled cells revealed detailed images of neuronal cell bodies, dendritic arbors and axonal projections (Figure 4). Dendritic tufts, a typical characteristic of horizontal cells, were also clearly visualized (Figure 4, Panels A–C).
The produced results are well within the quality range for morphological reconstructions or neurite mapping studies [3,15,16]. Moreover, we confirmed that the quality of the images obtained with the new illumination method is at the same level as that obtained with a mercury lamp microscope, as both methods yielded highly detailed stainings of the entire cell and its processes (Figure S3 and Table 1). However, a few requirements must be followed to obtain a high quality labeling: a) the DAB solution should be filtered before use; preferably with filters with a 40 μm pore diameter or less; b) the DAB solution should be periodically exchanged during the photooxidation process; we obtained optimal results by exchanging the solution every

Figure 3. Representative photomicrograph of a Cajal-Retzius cell stained by the new photooxidation method. A: Low magnification (40 × objective lens) photomicrograph of the DAB-stained Cajal-Retzius Cell obtained from a transverse section of developing rat neocortex. Note that the cell is stained over its entire volume, with no variations in staining intensity. The red arrow points at the cell’s axon. The blue arrow indicates the large horizontal dendrite that defines the Cajal-Retzius cell type. The orange arrow points at the cell’s soma. Scale bar corresponds to 20 μm. B: High magnification (100 × objective lens) photomicrograph of the insert indicated in A (dotted line square). Note the successful staining of dendritic shafts and spine-like appendages (green arrows), highlighting the high degree of detail and acuity of the staining. Scale bar corresponds to 10 μm.

doi:10.1371/journal.pone.0056512.g003

Figure 4. Representative photomicrographs of human retinal neurons and axons stained by the new photooxidation method. Cells were processed and observed in intact post-mortem human retinas. A and B: High magnification (100 × objective lens) photomicrographs of DAB-stained putative horizontal retinal neurons. Note the clear delineation of the cell body (blue arrows) and dendritic tufts (green arrows). Scale bars correspond to 10 μm. C: Low magnification (40 × objective lens) photomicrograph of two DAB-stained retinal cells (putatively horizontal cells). Scale bar corresponds to 20 μm. D: Low magnification (40 × objective lens) photomicrograph of a DAB stained axon terminal in a sample of human retina. Note that even fine structures, such as axonal knobs (arrows), are clearly stained. Scale bar corresponds to 20 μm.

doi:10.1371/journal.pone.0056512.g004
Methodological advantages of the new method

In addition to the already known advantages of photooxidation, including improved contrast and absence of photo-bleaching, our method makes the use of fluorescent microscopes unnecessary and allows for the simultaneous photooxidation of multiple labeled sites across large tissue samples, as it does not depend on the focal area of a microscope objective lens. These advantages make the method extremely cost and time efficient when compared with conventional photooxidation protocols, especially for large tissue samples, such as human retinas.

Wide-field illumination for fluorescent photooxidation

One of the main advantages of the photooxidizer apparatus is its relatively wide light beam in comparison to microscope-based illumination methods. We measured the effective illumination field of both a conventional fluorescent microscope and the photooxidizer by quantifying the area of tissue that was successfully photooxidized within a single reaction session (2–3 hours). The criterion for a successful photooxidation was the same in both experiments, i.e., the imaging of high resolution DAB staining in cell bodies and neurites. We found that the illumination field in both cases can be approximated to a perfect circle (Figure 5). The region of tissue that was successfully photooxidized with the fluorescence microscope was fitted within a circle with approximately 1.32 mm of radius and an area of 5.5 mm², while the region of tissue that was successfully processed with the photooxidizer was fitted within a circle with a radius of around 3.66 mm and area of 42 mm² (Figure 5). Therefore, the new photooxidizer apparatus is capable of processing an area of tissue over 7.5 times larger than what would be possible with currently available methods, all within similar time frames and quality standards. This methodological advantage may be of high value for the detailed investigation of relatively large cellular and histological structures, such as neuronal projection fibers and individual axonal collaterals.

Cost-efficiency of the new method

By eliminating the need for fluorescence microscopy, and consequently of mercury arc light bulbs, for the photooxidation reaction, the presented method provides a highly cost-efficient alternative to current protocols. This can be demonstrated by comparing the cost per hour of each methodological approach (summary in Table 1). Currently, high efficiency mercury arc lamps for fluorescence microscopy have a marketed average lifetime of approximately 2,500 hours and cost around US$500. This implies an estimated average cost of US$0.20/hour of lamp usage. Our apparatus, on the other hand, has a total cost of less than US$100, including the high power LED and all of its electronic and custom built components. Given that the high power LED we used has a 100,000 hour life expectancy, photooxidation with our method costs less than US$0.001/hour of usage. Our apparatus, on the other hand, has a total cost of less than US$100, including the high power LED and all of its electronic and custom built components. Given that the high power LED we used has a 100,000 hour life expectancy, photooxidation with our method costs less than US$0.001/hour of usage. In other words, we estimate the new method to be at least 200 times cheaper than current photooxidation techniques.

This cost difference becomes even more significant when one considers the advantages of wide-field illumination (Table 1). Considering the measured illumination field areas in the conventional fluorescence microscope (5.5 mm²) and the photooxidizer (42 mm²), the life expectancy of high-end mercury arc lamps for fluorescence microscopy have a marketed average lifetime of approximately 2,500 hours and cost around US$500. This implies an estimated average cost of US$0.20/hour of lamp usage. Our apparatus, on the other hand, has a total cost of less than US$100, including the high power LED and all of its electronic and custom built components. Given that the high power LED we used has a 100,000 hour life expectancy, photooxidation with our method costs less than US$0.001/hour of usage. Our apparatus, on the other hand, has a total cost of less than US$100, including the high power LED and all of its electronic and custom built components. Given that the high power LED we used has a 100,000 hour life expectancy, photooxidation with our method costs less than US$0.001/hour of usage. In other words, we estimate the new method to be at least 200 times cheaper than current photooxidation techniques.

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lamps (2,500 hours) and high power LEDs (100,000 hour) and the fact that a successful photooxidation reaction takes a maximum of 3 hours with both methods, we estimate that the maximum possible area of tissue photooxidized with a mercury lamp during its entire lifetime is approximately 4,583 mm², while the maximum possible area of tissue photooxidized with our system during the entire lifetime of a high power LED is 1,400,000 mm². By dividing the cost of the mercury lamp and the photooxidizer apparatus by these area estimates, we predict a lifetime cost per area of processed tissue of around US$0.11/mm² for conventional apparatus by these area estimates, we predict a lifetime cost per area of processed tissue of around US$0.11/mm² for conventional apparatus. 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Supporting Information

Figure S1 Representative photomicrographs of Dil-filled neocortical neurons before the submission of the tissue samples to the photooxidation reaction. Observe that the neuronal processes are well filled, a factor that is paramount for a good DAB staining in the subsequent photooxidation reaction. Scale bars correspond to 20 μm. A and B: Photomicrographs (40× objective lens) of neocortical neurons filled with Dil in samples of developing rat neocortex.

(TIF)

Figure S2 Representative photomicrographs of Dil-filled human retinal neurons before and after the submission of the tissue samples to photooxidation reaction with our novel method. Note that we were able to image the same group of cells under fluorescent microscopy (before photooxidation) and under light microscopy (after photooxidation). These images illustrate both the reliability of the method and the important advantage of photooxidation in general for studies of cellular morphology, given that the resolution for visualizing neuronal processes is greatly increased by this process. Colored arrows identify specific cells that are present in both panels. Scale bars correspond to 50 μm. A: Photomicrograph (20× objective lens) of neurons filled with Dil in samples of post-mortem human retina. B: Photomicrograph (20× objective lens) of the same neurons imaged in Panel A after submitting the tissue sample to photooxidation reaction with our novel apparatus.

(TIF)

Figure S3 Representative photomicrographs of DAB-stained human retinal neurons photooxidized with our new illumination method and with a traditional mercury lamp-based technique. Note that both illumination methods produce images of similar quality, i.e. they result in a highly detailed staining of the entire cell, including the dendrites and dendritic appendages. Scale bars correspond to 10 μm. A and B: Photomicrograph (100× objective lens) of DAB-stained human retinal neurons photooxidized with the novel photooxidizer apparatus. C and D: Photomicrograph (100× objective lens) of DAB-stained human retinal neurons photooxidized with a conventional fluorescent microscope.

(TIF)

File S1 Detailed schematic for building the photooxidizer. (PDF)

Table S1 Complete list of all commercial components of the photooxidizer apparatus. (DOCX)

Table S2 Complete list of all custom built components of the photooxidizer apparatus. (DOCX)

Acknowledgments

We thank Dr. Jan Kremers for his insightful comments and for critically reviewing the manuscript. We also thank Flavia G. M. Farias and Dr. Balázs V. Nagy for the technical assistance.

Author Contributions

Conceived and designed the experiments: MSF KMC. Performed the experiments: MSF DVVS. Analyzed the data: MSF KMC. Contributed reagents/materials/analysis tools: MSF. Wrote the paper: MSF KMC.

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