Anaglyph of Retinal Stem Cells and Developing Axons: Selective Volume Enhancement in Microscopy Images

NÉSTOR GABRIEL CARRI,[1]* SEBASTIÁN NOO BERMÚDEZ,[1] LUCIANO FIORE,[2] JENNIFER DI NAPOLI,[2] AND GABRIEL SCICOLONE[2]

[1]Laboratorio de Biología Molecular del Desarrollo, lnstituto Multidisciplinario de Biología Celular, IMBICE, La Plata, Argentina

[2]Laboratorio de Neurobiologia del Desarrollo, Instituto de Biología Celular y Neurosciencia "Prof. Eduardo De Robertis" (UBA-CONICET), Facultad de Medicina, Universidad de Buenos Aires, Ciudad Buenos Aires, Argentina

ABSTRACT

Retinal stem cell culture has become a powerful research tool, but it requires reliable methods to obtain high-quality images of living and fixed cells. This study describes a procedure for using phase contrast microscopy to obtain three-dimensional (3-D) images for the study of living cells by photographing a living cell in a culture dish from bottom to top, as well as a procedure to increase the quality of scanning electron micrographs and laser confocal images. The procedure may also be used to photograph clusters of neural stem cells, and retinal explants with vigorous axonal growth. In the case of scanning electron microscopy and laser confocal images, a Gaussian procedure is applied to the original images. The methodology allows for the creation of anaglyphs and video reconstructions, and provides high-quality images for characterizing living cells or tissues, fixed cells or tissues, or organs observed with scanning electron and laser confocal microscopy. Its greatest advantage is that it is easy to obtain good results without expensive equipment. The procedure is fast, precise, simple, and offers a strategic tool for obtaining 3-D reconstructions of cells and axons suitable for easily determining the orientation and polarity of a specimen. It also enables video reconstructions to be created, even of specimens parallel to the plastic base of a tissue culture dish, It is also helpful for studying the distribution and organization of living cells in a culture, as it provides the same powerful information as optical tomography, which most confocal microscopes cannot do on sterile living cells. Anat Rec, 00:000–000, 2014. © 2014 Wiley Periodicals, Inc.

Key words: neural retina; retinal stem cell; retinal progenitors; neurosphere; retinal axons; Eph-ephrin system; anaglyph; video reconstruction; Gaussian enrichment

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*Correspondence to: Néstor Gabriel Carri, MD, PhD, Laboratorio de Biología Molecular del Desarrollo IMBICE, CC 403, 1900 La Plata, Argentina. Fax: 54-221-425-3320.
E-mail: gabrielcarri@yahoo.se

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INTRODUCTION

The increasing use of stem cells means there is more need for microscopy techniques to obtain useful information through digital data processing. Three-dimensional (3-D) techniques are of great value for the study of cultured cells because they enlarge cell details and make their components more noticeable (Gustafsson et al., 2008; Le Baccon, 2012). However, one of the most important limitations in microscopic analysis of living cells is the inability to visualize the volume of the recorded object under sterile culture conditions. Further, processing micrographs in experimental biology is a significant issue, because the quality of the micrographs governs the ability to perform reliable analyses. 3-D photographs are among the most difficult to process and analyze, and methods for the high-quality processing of 3-D images would be very valuable (Solovei and Cremer, 2010; Shao et al., 2011; Swedlow, 2012).

The term "anaglyph" refers to a stereoscopic image that creates the illusion of depth in the visual cortex. This is done by encoding an image for each eye, using filters of chromatically opposite colors, commonly red and cyan. An anaglyph can highlight specific cell parts, and elucidate the volume of living sterile cells which is quite difficult to show in a two-dimensional (2-D) image (Fiore et al., 2010; Di Napoli et al., 2010a), making an anaglyph a useful strategic tool for embryology and cell biology.

We developed simple, fast, economical, and high-quality methods for obtaining 3-D reconstructions, including anaglyphs of living cells *in vitro*, as well as fixed and processed cells, axons, or tissues. Our method can gather and analyze information about 3-D specimens from crude micrographs generated using different types of illumination and processing techniques although important limitations persist. Ours procedures are simple to image depth and estimate volume. We present elaborate pictures of living stem cells, including anaglyphs, and good illustrative full depth-of-field images of living retinas, growing axons, and differentiating glial cells, and show that the behavior of neural stem cells under special culture conditions can be imaged. These straightforward, rapid, and precise methods could be applied to any cell in culture or to embryonic samples.

The method for creating 3-D images of living cells using phase contrast illumination is shown in the section "**Living Cells, Neurospheres, and Explants**". In the section "**Fixed Retinal Neurospheres and Retinal Axons**," we illustrate the procedure of applying an algorithm for a Gaussian blur filter to focused images to enrich the details of electron micrographs and confocal images.

MATERIALS AND METHODS

Embryonic Tissues

Chick embryos were obtained from fertilized White Leghorn (*Gallus gallus*), pathogen-free eggs, from the Rosenbusch Institute (Buenos Aires). They were incubated at 100°F (38°C) and 60% of relative humidity. Developmental stages were stated according to Hamburger and Hamilton (HH) (1951). Embryos were removed from the eggs, decapitated, and dissected in ice-cold Hank's Balanced Salt Solution. For experiments with living stem cells, the retina was removed with our punched-out neural tissue procedure (Carri and Ebendal, 1986). The outgrowths were grown from the chick retinal ciliary marginal zone isolated from eyes of embryos at different developmental stages from day 3 (HH19-20) to day 9 (HH 35). For the axonal immunostaining experiments, these were from day 7 (HH 30-31) embryos prepared as described previously (Ortalli et al., 2012).

Instruments and Procedures

All steps were performed using routine lab protocols under sterile conditions in a laminar flow cabinet with the aid of a stereo microscope illuminated with fiber-optic conducted light (Schott light source KL 150). Surgery, cell disaggregation, and manipulation in culture were performed using an apparatus consisting of a capillary connected by silicone tubing to a Wellcome Syringe®. This is essential for handling small pieces of retina with the required orientation and limited trauma (Carri and Ebendal, 1984, 1986). Briefly, the interior part of the eye was exposed by removing the distal half of the ocular bulb, and selected plugs of peri–iris neural retina were obtained from the proximal half.

For cell preparations, explanted tissue was washed twice and mechanically disaggregated. Cells were cultured, floating in stem cell medium in a 50-mL plastic flask in a humidified atmosphere at 37°C and 5% of CO_2 for 24 hr (Cruz Gaitán et al., 2010). This procedure produced retinal neurospheres. After 48 hr in the flasks, the cells were transferred to a 100-μL drop of medium in a 35-mm plastic culture dish precoated with Type I collagen. Cells were then kept in the same trophic medium and observed for weeks, taking pictures at different time points.

Organotypic plugs with stem cells were obtained using the capillary apparatus described above. The apparatus was placed on the left-hand side of the microscope and explants were manipulated by repeated aspiration into and expulsion from the capillary and finally placed in the culture dish. The position of the explants was determined by the characteristics of the niches explanted. Explants were used for studying the stimulation of outgrowth produced by the trophic activity of mitogenic and differentiation factors. The spatial architecture of the plugs was maintained, and they were placed on collagen gels prepared as described earlier (Elsdale and Bard, 1972). Trophic stimulation of the outgrowth was produced by the addition of 20 ng/mL of glial-derived growth factor (GDNF) from Peprotech, USA.

Living Cells, Neurospheres, and Explants

Image capture, camera, and microscope. Pictures were obtained with a Digital D-100 Nikon camera mounted on a Zeiss IM35-inverted microscope, using a tungsten lamp and phase contrast illumination. Image series for cells were taken with the following objectives: an F-LD 32× (na: 0.4, Ph1), for neurospheres with an F10× (na: 0.25 Ph1) and for retinal outgrowth explants with a 2.5× (0.08, from Leitz). The manual mode with sharpness criterion was used for image focussing. Micrometer has a resolution of 1.38 μm in each line. After selecting the specimen, objectives were focused on the cell detail nearest to the level of the plastic dish (0 z level, Fig. 1). Then photographs were taken up regularly at 6.9-μm intervals, ignoring the focus and using the remote button to diminish camera shaking (Figs. 2–7).

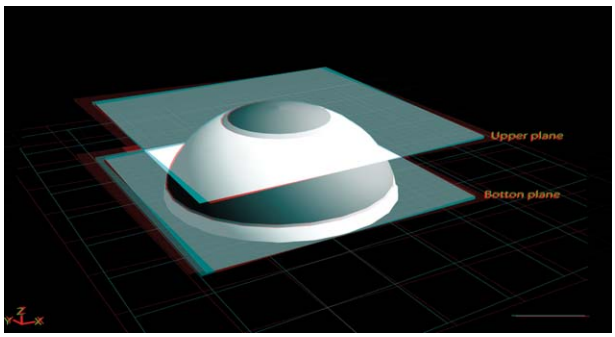

Fig. 1. A schematic view of an object to show the *z*-level at which the photograph was taken with a camera fixed to the microscope. These frames give enough levels (20 photos) to recreate volume with the reconstruction procedure used to produce the final image. A diagram of *x*-, *y*-, and *z*-planes is shown in the bottom-left corner. Imaginary planes cut the object at the bottom level where the object is attached (the plastic culture dish) and the upper plane opposite to the plastic. Bottom-right corner, the magnification bar is 33 μm.

The final focus of the image will be then the software integration of the 20 images taken every 6.9 μm to cover 138 μm; for example, the height one of the neurosphere. These constituted a complete set of photographs numbered from 1 to 20 from the bottom to the top.

From photo series to fully integrated image. The captured images were processed using Helicon Focus 5.1® software (Helicon Soft, United Kingdom). Each photographic frame was identified with a number from 1 to 20, from the bottom to the top of the object as described above (Fig. 1). The Helicon Focus 5.1® support software is compatible with several RAW formats, including JPEG, TIFF, BMP, and others. We used TIFF format. These files were then kept numbered in a folder and loaded for processing in the software. With this series of photos, we were able to reconstruct a good detailed image, and the overall focus was based on the interpolated information of the levels used. The software processing utilized a statistical algorithm in the Helicon Focus 5.1® which deciphers differences and similarities between the various components of the images. Results from this algorithm were then analyzed by simple data comparison according to the final spatial distribution.

The final result was the exact *z*-value for each pixel in the *x*/*y* plane which, by means of trigonometric calculations, was used to generate a set of polygons that served as the skeleton for the 3-D image processing unit of Helicon Focus® software 5.1. From this 3-D model, an anaglyph was constructed using two different capture positions of the software which, when finished, provided a stereoscopic view of the object with excellent depth-of-field. Additional details for individual image series are provided in the figure captions. The software can be used for processing a set of images to generate an overall focused image, or to show the final black and white or full color anaglyph.

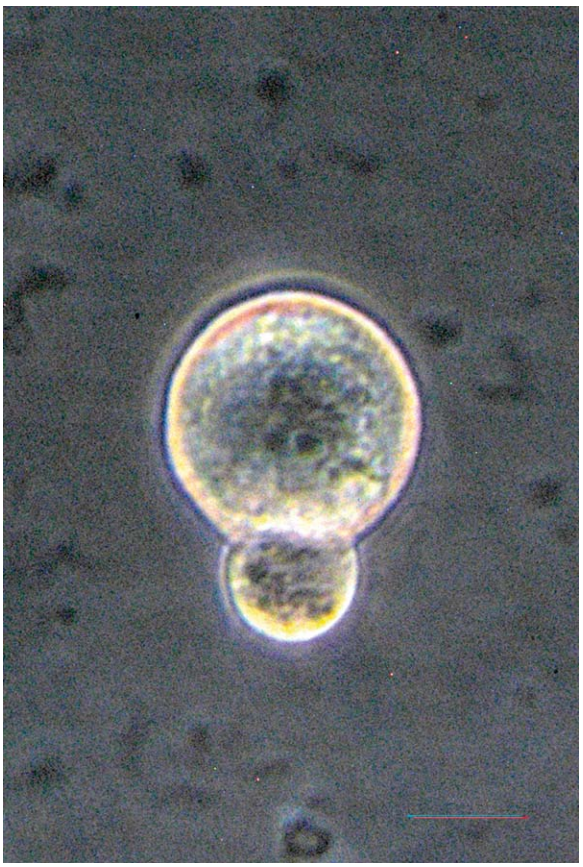

Fig. 2. PLATE A – Image of living retinal stem cells after mitosis on Type I collagen monomer coating on plastic culture dish. The high-quality focus is owing to the composition produced by the software integration of 20 standard photographs focused from the bottom to the top. The cells were observed for recording a mitosis in which two subsequent cells of different sizes were generated. Phase contrast illumination with 32× objective (Ph1). Magnification bar, 12 μm.

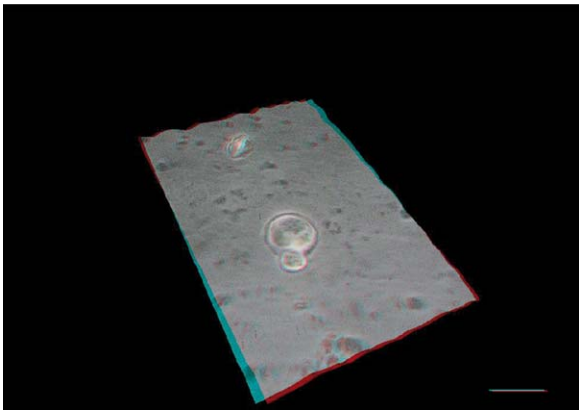

Fig. 3. PLATE A – Red–cyan anaglyph of the same living retinal stem cells shown in Fig. 2. The image shows well-preserved cell morphology with good cell volume owing to the success of the procedure. Phase contrast illumination with 32× objective (Ph1). Magnification bar, 25 μm.

Fixed Retinal Neurospheres and Retinal Axons

The embryonic retinal neurospheres were processed as dry metallized specimens with routine protocols for scanning electron microscopy. Retinal axons were processed as wet double immunolabeled samples, covered with Fluoromount™ (Southern Biotechnology, Birmingham) mounting medium.

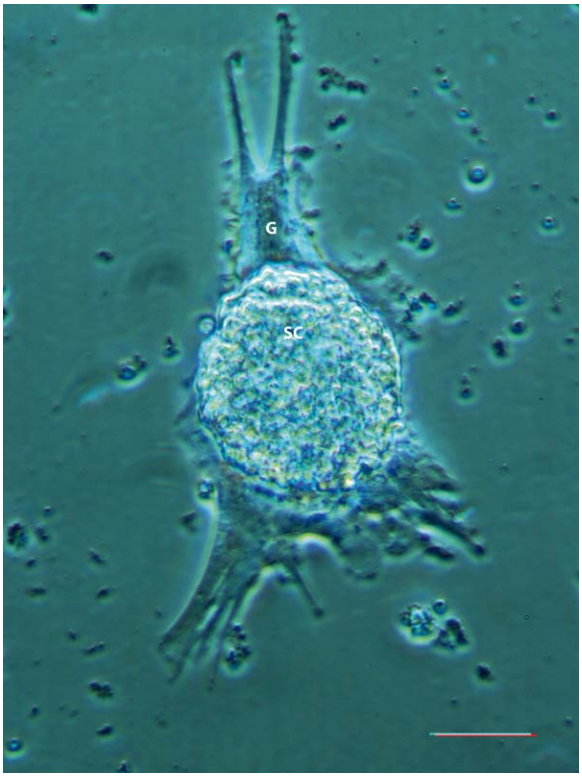

Fig. 4. PLATE B – Image of the living retinal neurosphere attached to Type I collagen monomer coating the plastic culture dish. The high-quality focus of the sphere is owing to the integration of 20 standard photos of the cell as shown in Figs. 2, 3. The neurosphere was cultured for 1 week and observed for recording glial outgrowth (G) generated from the central retinal stem cells (SCs). Phase contrast illumination with 10× objectives (Ph1). Magnification bar, 130 μm.

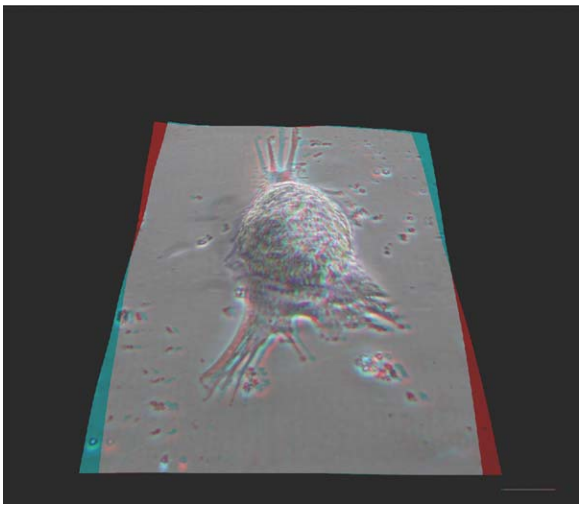

Fig. 5. PLATE B – Red–cyan anaglyph of the same neurosphere shown in Fig. 4. As stated in Fig. 4, the neurosphere was cultured to observe the glial outgrowth generated from the central stem cells. The image shows the structure of the stem cells sphere with great detail, thanks to the enhancement of volume achieved through reconstruction, so that it looks as if a snail was moving over the glial cells. Phase contrast illumination with 10× objective. Magnification bar, 120 μm.

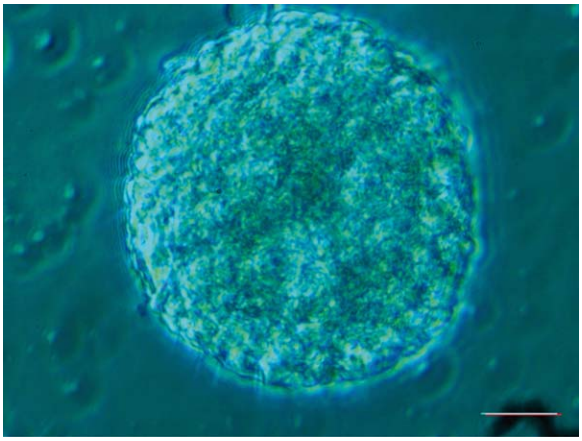

Fig. 6. PLATE C – Image of the explant of neural retina on collagen gel substrate. The high-quality focus also results from composition by software integration. The explant was observed for recording of cell migration and tissue outgrowth. Photograph was taken 1 hr after implantation onto the gel. Phase contrast illumination with 2.5× objective. Magnification bar, 250 μm.

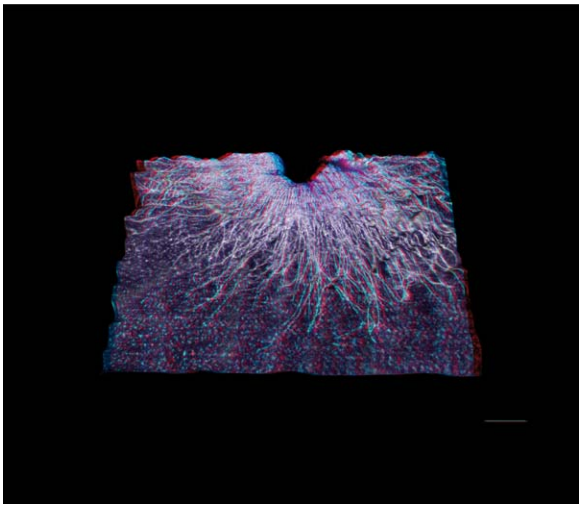

Fig. 7. PLATE C – Red–cyan anaglyph of the living neural retina explant shown in Fig. 6. The explant was cultured for 4 days on collagen gel to observe neuronal response to trophic factors such as an outgrowth generated from the retinal ganglion cells. Very low magnification with dark field illumination at 2.5× objective. Magnification bar, 1,000 μm.

Retinal stem cell spheres. Retinal cells, corresponding to a living neurosphere floating in culture media, were fixed and processed for scanning electron microscopy, applying conventional technical protocols. Briefly, after rinsing with PBS the specimens were washed in 0.1 M of cacodylate buffer, pH 7.4. Neural stem cells (neurospheres) were fixed in 2.5% of glutaraldehyde in 0.1 M of cacodylate buffer, pH 7.4, at room temperature. After 30 min, the neurospheres were carefully removed from the culture dish and stored for at least 1 day in the same fixative containing 0.1% of eosin. Eosin-staining during fixation helped to identify the structures, mainly the smallest specimens, with only a few cells. The specimens were then washed for 1 hr in a

0.1-M cacodylate buffer, pH 7.4, refixed in osmium tetroxide, and dehydrated in graded acetone solutions. The dried specimens were then mounted on microscope holders with conductive silver paint and successively covered with carbon and gold–palladium coats in a rotator evaporator.

Scanning electron microscopy. The observation of retinal neurospheres was done in a Zeiss Supra 35VP X-Edax Gemini scanning electron microscope operated at 5 or 15 kV for biological specimens. The focus was adjusted manually or automatically. Neurosphere images were kept, divided into several pictures, in a file holder.

Retinal explant cultures. Seven-day-old embryos, HH 30–31, were removed from the eggs and processed as previously described by Ortalli et al. (2012). The eyes were enucleated, and the cornea, lens, marginal zone, and vitreous humor removed and discarded, exposing the central neural retina. The neural retina was separated from the retinal pigment epithelium under a stereo microscope. The edges of the optic vesicle were partially cut with a surgical microscalpel for orientation. Each retina was divided into three sections from nasal to temporal pole; the middle third was discarded and the nasal and temporal regions were then cultured in N2-supplemented F12/DMEM containing 0.4% of methylcellulose (Sigma, St. Louis, MO) on coated coverslips placed in culture dishes as described earlier (Ortalli et al., 2012). Coverslips were coated with poly-L-lysine (200 μg/mL) (Sigma) and laminin (20 μg/mL) (Invitrogen, Grand Island, NY) for 2 hr at room temperature.

EphA3-Fc fusion protein (R&D Systems, Minneapolis, MN) or Fc (R&D Systems) was clustered with an antihuman Fc polyclonal antibody (Cappel-MP, Santa Ana, CA). EphA3-Fc, the antihuman Fc antibody and EphA3-Fc, or Fc, were preincubated for 1 hr at room temperature, and then added at 0.25 μg/mL (2 nM) Fc to the retinal explant cultures. Explants were cultured in a humidified atmosphere at 37°C and 5% of CO_2 for 24 hr in the dark. Then, the cultures were fixed with paraformaldehyde 2%–sucrose 2% in PBS for 30 min at room temperature (Scicolone et al., 2009; Fiore et al., 2010; Di Napoli et al., 2010a). The retinal axons to be shown on anaglyphs were obtained from these cultures.

Axonal immunohistochemistry. For immunofluorescence, nonspecific binding was blocked by preincubation with 5% of normal goat serum in PBS with or without 0.5% of Tween-20 (Sigma) for 1 hr, and then incubation with the primary antibodies for 30 min at room temperature. For double immunostaining, two antibodies were added at the same time. The following primary antibodies were used at 1 mg/mL: (i) rabbit antiephrin-A2 (L-20, sc912) and (ii) mouse anti-EphA4 (S-20, sc921) (Santa Cruz Biotech). These were diluted in PBS containing 2% of normal goat serum with or without 0.5% of Tween-20. A negative control was made by the omission of the primary antibody. The specimens were then incubated with Alexa Fluor 488 (green)-conjugated F(ab′)2 fragment of goat antimouse antibody (A-11020, Molecular Probes, Eugene) and Alexa Fluor 594 (red)-conjugated F(ab′)2 fragment of goat antirabbit antibody (A-11072, Molecular Probes) (Dempsey et al., 2012). Finally, they were mounted with Fluoromount-G (Southern Biotechnology).

Confocal laser microscopy. Confocal images were acquired with an Olympus FV300 microscope (Tokyo, Japan) using a green Ar laser 488 nm, 10 mW, Red HeNe laser 633 nm, 10 mW with z-depth of 1.4 μm. Exposure and gain of laser were the same for all conditions in each experiment. Ten distinct fields of axons growing from explants were acquired for each condition. Seven photographs per field were obtained along the z-axis. One plane of section was selected and photographed, and then three successive planes were photographed above the first one and another three successive planes below it. Images were taken at 0.2-μm intervals on the z-axis.

Image acquisition, processing, and final reconstruction. It is very difficult to reconstruct volume in a series of images of neurospheres and axons. Our procedure consists of five different stages outlined below, culminating in a final reconstruction that gives the final 3-D model, the tubular or spherical shape, most similar to the actual biological shape (flow chart, Fig. 8; the results, Figs. 9–14).

Stages. i. Imaging—A series of 2-D *xy* pictures are obtained with a digital camera from a microscope just by changing focus for different levels of *z*.

ii. Processing—Each series is easily integrated into a digitally assembled image using the Helicon Focus 5.1® software. Technically, there are two possibilities when applying the software: method A permits more defined images, whereas method B provides images with more detail in the depth map generated by comparing pixel by pixel. The operator must choose the appropriate method for the sample acquired, based on the morphology of the sample. This second processing stage integrates a series of images into a secondary overall focused one (Fig. 8).

iii and iv. Layering and Gaussian Blurring—These two processing steps create different levels of information, through layering of the image, using editing tools from Adobe Photoshop C5® software (Seattle). The tools are for processing digital images to separate their elements which, in our samples, are cells from the background or axons from the substrata. In these steps, three different levels of information, *x*, *y*, and *z*, are used for creating the layers. In each layer, *z*-information is improved, keeping image details. The resulting virtual layers can then be superimposed on each other, making it possible, for example, to group or discriminate image compounds or mix different forms of light and color combinations (Fig. 8). A "mask" is linked to a hidden layer and another (transparent) part that reveals the bottom of the image layer. This "masking" method is applied to hide the gray areas, overlapping another layer that contains the same information for that position, but in more detail in the photo.

The following sequential processing steps were carried out on the image of fixed tissues in Adobe Photoshop C5® software:

1. Open the image;
2. Select the sample with the polygonal lasso tool to mark the outline of a neurosphere or the axons;
3. Copy them to a new layer (Copy and Paste);

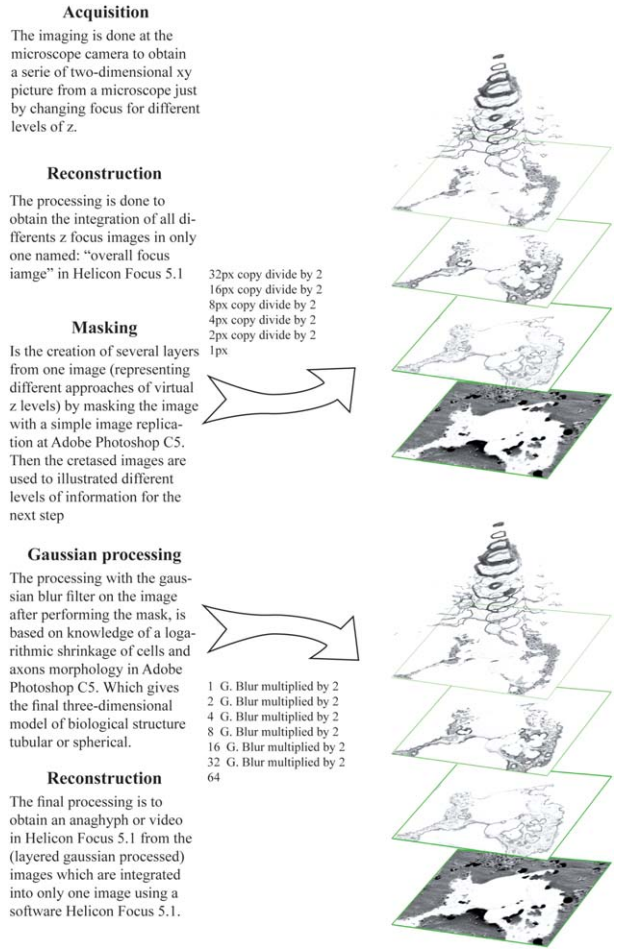

Fig. 8. PLATE C – Schematic illustration of the acquisition, focus reconstruction, masking, Gaussian processing and anaglyph reconstruction on the left-hand side of the scheme. Layerization and Gaussian treatment applied to neurosphere shown in Fig. 9 are illustrated at the right-hand side of the scheme. Layerization is done for creating layers on the image that represent different levels of focus, and is shown in the upper part. Gaussian blur, to correct the focus of the image is shown in the lower part of the scheme. Both are processed in Adobe Photoshop C5®.

4. Hide the background;
5. Capture the selection by pressing Control + click the layer icon figure;
6. Select "select" in the menu, go to "get selection," and put in the value of 32 pixels;
7. Copy the selection to a new layer (Copy and Paste);
8. Hide the previous layer;
9. Repeat Steps 5–8, reducing the number of pixels evaluated from the previous layer each time, dividing by 2;
10. Proceed to the next step when the reduction of the number of pixels reaches the value 1;
11. Select each individual layer to apply Gaussian blur. Increasing blur should increase from the first layer at 0 (full focus) to reach 32 pixels at the bottom layer, multiplying the previous Gaussian blur by 2, that is: 0, 1, 2, 4, 8, 16, 32, 64 from 0 to the last layer, respectively (Fig. 8).

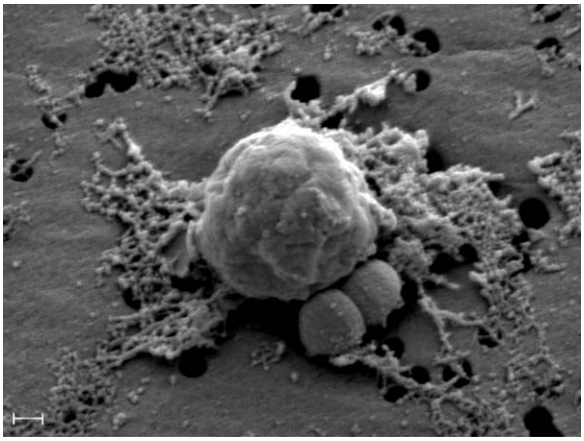

Fig. 9. PLATE D – Electron photomicrography of a fixed-dry neurosphere of retinal stem cells shown on the membrane used for handling the specimen during scanning preparation (critical point dry system and metallization). The neurosphere was cultured for 10 days before fixation. Scanning electron microscopy. Magnification bar, 40 μm.

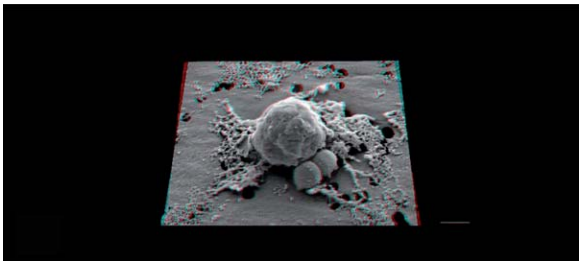

Fig. 10. PLATE D – Anaglyph of the same image shown in Fig. 9 rendered by Helicon Focus 5.1® software after treatment with Gaussian blur filter. The spatial volume of the neurosphere is owing to the reconstitution of the 14 planes created with Gaussian treatment through Adobe Photoshop C5® software. Note the number of cells in the upper part of the neurosphere. Magnification bar, 40 μm.

12. Save the composition as Adobe Photoshop C5® to keep layer information;
13. Save the resulting image again as a TIFF file for processing, with the name of the first image;
14. Open the file in Adobe Photoshop C5® with the layer information saved;
15. Repeat Step 11 with a blur value of 1 for the first layer, followed by 0, then 1, then 2, and continue as it was explained above. The values increase by multiplying the previous value by 2. Repeat the procedure in a row with eight layers, that is: 1, 0, 1, 2, 4, 8, 16, and 32 (Fig. 8);
16. Save the resulting image as a TIFF file with the name of the first image; and
17. Repeat Steps 14–16 for the application of the values in the list (image), but in each change of cycle reposition the value 0 in the next layer until the last layer.

As a result, we get a series of image files with layers with different virtual focuses at several levels on a logarithmic scale of imaginary histologic sections of the tissue sample.

v. Creating anaglyphs—An anaglyph can be created by digitally assembling reconstructed modified images in

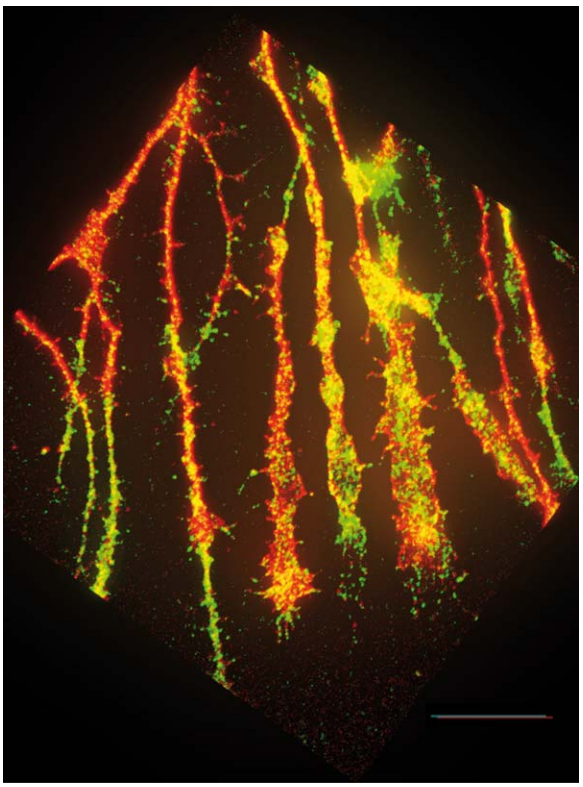

Fig. 11. PLATE E – The confocal image of fixed retinal axons immunostained with two different antibodies, anti-ephrin-A2 in red and anti-EphA4 in green. Epi-illumination with 60× objective. Magnification, bar 10 μm.

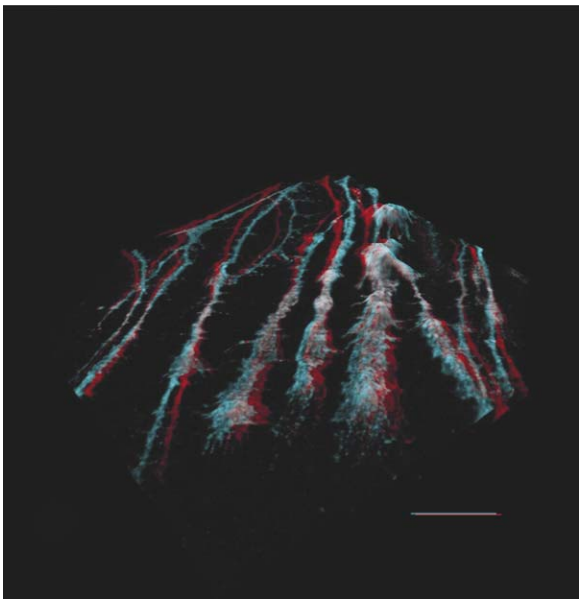

Fig. 12. PLATE E – Anaglyph of image shown in Fig. 11 after reconstitution in Helicon Focus 5.1® software. Note that the filopodias and lamellipodias of the growth cone are more clearly appreciated in stereology of a 3-D image. Growth of these retinal ganglion cell axons is guided by a gradient of EphA3-Fc. Epi-illumination with 60× objective. Magnification bar, 10 μm.

Helicon Focus® to obtain final 3-D model (Fig. 8 and the results in Figs. 10, 12–14).

RESULTS

Procedure for Improving the Images of Cells in Culture

The procedure described here for making a stereographic reconstruction from serial photographs of living cells proved an excellent tool for analyzing cultured embryonic tissues (Figs. 2–7). Floating cells were imaged immediately after mitosis (Figs. 2, 3) as they were attached cell cultures which showed a magnificent outgrowth of cells (Figs. 4, 5). In addition, we obtained images of an organotypic explant at the moment of implantation into collagen gels, and after a vigorous GDNF-stimulated axonal outgrowth (Figs. 6, 7). We reconstructed images of cells (Fig. 3), an attached neurosphere (Fig. 5), and an organotypic explant (Fig. 7) from serial pictures taken after treatment with trophic factors. In addition, we reconstructed images of other embryonic tissues such as neurospheres from brain cells or embryos (data not shown).

It was also possible to reconstruct these floating objects into an anaglyph. The texture and shape showed that there are no distortions owing to soft-processing. This can be appreciated as shown in Figs. 6, 7. One of the limitations of our procedure is the difficulty of working with super-flat cells in culture, such as retinal Müller cells.

Culture models are frequently used to assay the behavior of stem cells or their physiological response to different trophic treatments (Figs. 4, 7), and our image enhancements make it possible to better visualize responses to treatment. An anaglyph constructed by our method (Fig. 5) made it possible to distinguish between neurons and neural glial cells and to visualize a snail-like neurosphere which appeared to be crawling over the glial cells. It should thus prove possible to extend the use of this procedure to a number of different purposes in basic tissue culture, such as microimplantation, and so on. Reconstructions and videos are being made using this method for other short-term bioassays.

This method is exceptionally useful for analyzing the morphology and organization of living cells within a culture, because it provides the tomographical information of an optical image (Fig. 5) that the majority of confocal microscopes cannot produce without epi-illumination. Such information can be obtained, for example, from the image series of living cells (Fig. 2), living neurospheres (Fig. 4), and retinal explants (Fig. 6).

Process of Gaussian Enrichment of Scanning and Retinal Axon Images

We have included a stepped procedure for enhancing images into 3-D views with good volume rendering, clear cellular details, and highlighted components. The procedure was employed to reconstruct the images of immunostained retinal axons with a laser confocal microscope (Figs. 11–14). It was also employed with scanning electron photomicrography of dry-fixed metallized neurospheres (Fig. 10). Both axons and neurospheres were included for Gaussian processing. We have described a procedure for creating a complete serial set of images,

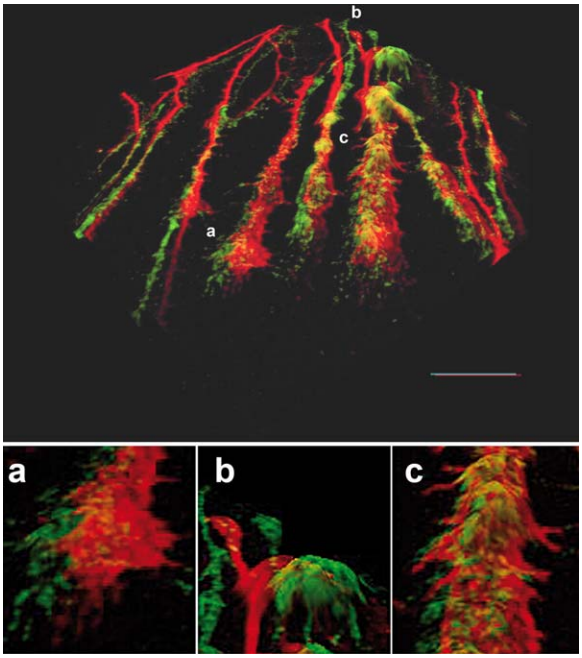

Fig. 13. PLATE F – The merged confocal images shown in Fig. 11 give a "pasty" morphology of the axon in which the two antigens identified by secondary red and secondary green are confused in a blurred yellow by the digital conjunction of two primary colors. Red and green secondary antibodies always give a well-defined cell structure with a precision of details that the resulting yellow mixture never reaches. It is necessary, if possible, to have an image that provides a comparative individual red and green color reconstruction of the antigen labeling on the axon morphology, that is, a way the red–green confocal double-detection can be reconstructed in Helicon Focus 5.1® with low angulations as option. Our procedure makes it possible for the optimized colored anaglyph to separate the merged yellow into its structure of red and green, enabling the exact correlation of each color *in situ* at the spatial positions on the axon morphology. Insets: The low-angled anaglyph gives our procedure the possibility of optimizing the separation of yellow into a clear red and green on the axon morphology (observ Fig. 11 and then compare the insets on the right (**a**), medial (**b**), and left images (**c**)). These are retinal axon structures outgrowing the explants with small and medium filopodias growing lateral to the main branch and also the lamelipodias and filopodias in the growth cone. These axons grow oriented from the retina to the EphA3-Fc distribution.

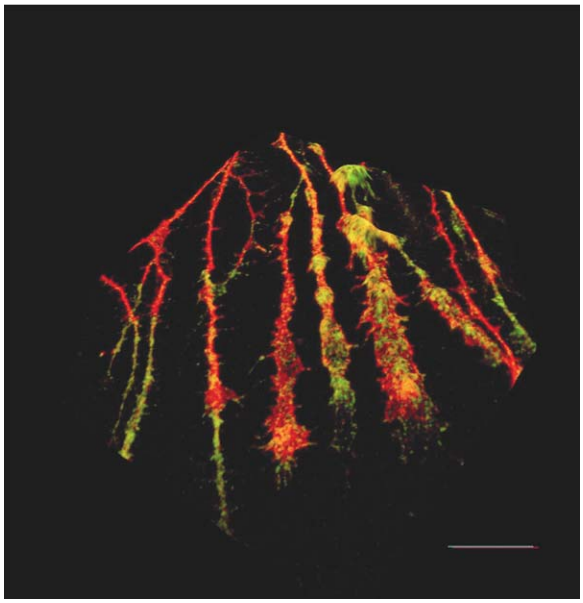

Fig. 14. PLATE F – Figure 11 after reconstitution with no angle at all.

which can be used for the reconstruction of stereographs and videos.

In both cases, 10–15 photographs were taken serially from the bottom to the top using a scanning electron or laser confocal microscope, but this was not enough to produce a deep anaglyph or video. This was done by applying a Gaussian blur filter on a digitally layerized set of images (Fig. 8). Each primary image from a scanning or confocal microscope was treated individually, but these series of primary acquisitions did not allow us to obtain the expected 3-D volume with the software (reconstructions not shown). The secondary images obtained from fixed specimens at the SEM, or with confocal microscopes, have more restrictions than the photographs of living cells obtained with phase contrast illumination as shown in Figs. 3, 5, 7. Scanning electron microscopy gave us a good spatial image with good resolution of the clumped cells, the surface of the cells, and the contacts between them; it also showed mitosis in the external ones and the spatial distribution among the whole group (for details, see Fig. 9). However, the usefulness of these images is limited by the restricted focus which could be obtained, manually or using autofocus. The photographs of these series are not suitable for reconstruction.

The treated images obtained with our proposed method produced excellent videos and stereo images (Fig. 8). Confocal microscopy also provides good images of double immunostaining (red and green) in a serial set of laser images of retinal ganglion cell axons, the growing of which is selectively stimulated by the EphA3 ectodomain (Fig. 11). Figure 11 is a representative example of the 10 fields photographed in one condition. In this case, we had difficulties recognizing the deep focus in the reconstructed anaglyph. It is almost impossible to enrich volume in anaglyphs from 2-D 16-bit TIFF images that have to be generated from the *z*-stack from sequential image files captured with two laser illumination, changing the beam focus over the axons (Fiore et al., 2010). We applied our Adobe Photoshop C5® layerization and Gaussian blur procedure to the confocal images obtained with red–green illumination to reconstruct the series with the Helicon Focus 5.1® software. The procedure generated clear enhanced 3-D images and corresponding 3-D video animations of retinal stem cells (Figs. 12–14 and Supplementary video).

DISCUSSION

Finest Images of Living Cells

Various types of cell culture models are frequently used to assay the effect of trophic factors on stem cells; one way of measuring this effect is to analyze the behavior of cells such as mitosis and migration, and also the physiological responses to these treatments, such as differentiation or apoptosis. The procedure described here provides an excellent tool to aid analysis.

Microscopists in tissue culture laboratories know the difficulties of obtaining high-quality well-focused micrographs of living objects (cells, neurospheres, or explants) (Pertusa Grau 2003, Toomre and Bewersdorf, 2010; Marubashi et al., 2011). These difficulties are in direct proportion to the objective used. Long distance objectives provide more resolution. However, less depth-of-field enables the easy reconstruction of the *z*-depth as shown in Fig. 5. Although it is possible to enhance the volume of photographed objects using other procedures, we were unable to obtain quality-standardized images such as those shown in Figs. 3, 5, 7 as rapidly and as easily as with the method described here. This methodology allowed us to quickly obtain the detailed photographs, impressive anaglyphs, and good videos from any living cell, neurosphere, or explant in culture, providing high versatility with respect to the size of the samples and the quality level of the reconstructions.

Multiple focus and the reverse engineering of images greatly improved their quality. The volume of living objects is an important property that is quite difficult to evaluate in routine work, but it may be rapidly and easily determined by means of this procedure. The procedure of reverse engineering the photographs of living cells described above provides an excellent tool for studying cells in several types of embryonic stem cell models (Fig. 1).

It is very difficult to store information on the behavior or responses of cells *in vitro* or *in vivo* in a video reconstruction. Microscopy of tissue culture techniques is always limited by long-distance objectives or by illumination of the specimen. But these limitations did not prevent us from getting the necessary information for the reverse engineering of images with software (Figs. 2–5). After the image reconstruction, we were able to identify unrecognized cellular details in the images and videos, as well as in the anaglyphs. This method could be extended to microimplantation or other tissue culture uses. As simple as it is, this procedure can become a strategic tool for embryology and cell biology.

Superior Scanning and Confocal Micrographs

As populations of stem cells routinely must be amplified *in vitro*, cell culture is a crucial technique. Phase contrast microscopy of living cells is useful during the preparation of the developing cells (Haug et al., 2009; Toomre and Bewersdorf, 2010). However, after cell enrichment, specific markers are required for studying them. Cells are themselves analyzed by laser confocal and scanning electron microscopy and reverse engineering is then used to construct stereoscopies of selected areas. Stem cell technology then requires an appropriate microscopic analysis of structural details, which demands a combination of powerful optical imaging and molecular labeling (Pertusa Grau, 2003). As van Teeffelen et al. (2012) clearly state, fluorescence microscopy is the primary tool for studying complex processes inside individual living cells.

The anaglyphs and videos of these fixed biological assays are of a quality similar to those of the living cells (Figs. 9–12). This method could provide good 3-D images for future industrial needs in which anaglyphs could be helpful. The present procedure is a simple and easy way to analyze cell behavior. It shows the data that are not available from microscopic images. Our underlying hypothesis is that data obtained from bi-dimensional 16-bit TIFF *x* and *y* references can generate a real *z*-stack of images which can be used to render stereography and videos. We tested our hypothesis by examining different dry or wet specimens with scanning or laser confocal microscopy. Anaglyphs and videos are digitally assembled secondary images reconstructed from primary photographs. In both cases, a series of 10–15 photomicrographs taken from the bottom to the upper part did not render good anaglyphs. Constructing secondary images is more complicated than taking a series of photographs while adjusting the focus in phase contrast illumination, as it was done in living cells.

Advantages and Disadvantages of the Helicon Focus 5.1® Software

The use of our method with this software significantly improved the quality of images from cultured living cells, which had to be photographed while maintaining culture sterility conditions. Cells and spheres in culture dishes have several spatial planes. Quality images of these cells or spheres can only be obtained if focus is set to infinity, losing angles, brightness, sharpness, and details. The application of the software to the series of micrographs provides an overall sharp and well-focused specimen image, minimizing defects from culture conditions. Our method with the software was designed to enhance images during routine work at the stem cell laboratory. One of the great advantages of this application is the sense of depth that normal illumination of the microscope does not offer. Moreover, it is impossible to achieve the virtual sense of volume from real-time phase contrast illumination of living cells. With this simple application, cells, neurospheres, and outgrowth can be observed in real, near to culture conditions, in a very simple way. No sophisticated or expensive equipment is necessary to obtain quality images of experimental results under tissue culture conditions. However, the software has some limitations: it cannot be automatically run, and contrast and brightness have limited commands from the software options.

Comparison

A comparison with other possible strategies that enhance a good overall focus for living cell assays, depth, brightness, and sense of volume of the cells would point up the importance of the method. However, we have not found any simpler and cheaper way to obtain these quality images from our cells, spheres, or stimulated explants with vigorous outgrowth. We tested different illumination angles trying to enhance shadows by following other criteria (Chandraker et al., 2007), and even simulated the virtual reflected-light microscopy published by Harrison (Harrison, 2010; Harrison et al., 2011), but we did not succeed, possibly because of the limitation of the epi-illumination. Image quality was not satisfactory because the resolution over this sphere required a powerfully transmitted light.

Several circumstances in our lab strongly support the application of our method. The clump of cells forming a neurosphere in culture, under sterile conditions and microscopic limitations, is opaque and does not allow light to pass through the specimens easily. It is difficult to

illuminate clumps of cells and capture transmitted light to make a 3-D image. Refinements for processing depth and brightness of the birefringence of living cells are always significant. We compared the same cell clump shown in Figs. 4, 5, which is the same sphere taken with bi-dimensional *x–y*, and then with the corresponding 3-D stereography. The processing affects the quality of the results. We subsequently discussed the performance of other approaches to evaluate our method. The virtual soft texture of the cell clump volume presented as a neurosphere was similar to that observed with SEM. Our proposal is a computerized tool to get high-quality images from experimental results, obtained from the refocused reflectance properties of phase contrast illumination. As an anaglyph, it is a reshape of a set of series of specimen images.

Gaussian Application

Scanning electron microcopy images are generated by acquiring a set of serial images while automatically or manually changing the focus of the beam on the sample. These serial photographs can then be integrated into a single one with the help of the Helicon Focus 5.1® software to produce a 3-D representation or anaglyph. This figure shows discrete volume and depth. However, this procedure suffers from notable noise in the reconstruction of serial frames, as it is easy to observe, for example, in the magnification bar automatically placed by the microscope on the picture. In images where the focus is programmed, the noise in the magnification bar goes down but the specimen is not so clearly focused. To solve this problem, we performed layerization and then Gaussian blur processing in Adobe Photoshop C5® to have more detailed reconstructions of individual pictures (see complete processing in Fig. 8 on the right-hand side of the scheme).

The serial set of images compiled from scanning electron microscopy was used to reconstruct an image with good multiple-focus through Helicon Focus 5.1® processing. This image was layerized with Adobe Photoshop C5® and then treated with a Gaussian blur algorithm with individual filter processing (the right-hand side of the scheme, Fig. 8). Then processed images were used to render the final anaglyph reconstruction, showing the volume of the ball. The depth and volume of the sphere are impossible to show with routine lab procedures. Gaussian processing creates steady images and allows us to process high-quality pictures in 3-D (Fig. 10) and also to render videos. The merged confocal pictures of retinal axons give overlapping areas of a soft yellow color (which represents the merging of the red and green antibodies) (Fig. 11). The application of triple reprocessing in our procedure, layering, filtering, and rendering, clearly increases the red and green details of the axon morphology (Fig. 12A). This reconstruction of processed materials makes it possible to separate the red and green components of the merged soft yellow color *in situ* with precise spatial positions in the axon morphology (Figs. 13, 14). Hence, small and medium-sized filopodia, growing laterally from the main axonal branch, and growth cone lamellipodia can be seen with their real 3-D morphology.

Future Work

We are testing the use of our stored data to provide comparative information on video reconstruction, with special attention to the texture of specimen immunostaining, which is quite difficult to visualize with laser confocal techniques. Our method is advantageous when compared to possible volume and depth renderings using other applications.

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LITERATURE CITED

Carri NG, Ebendal T. 1984. Stimulation of retinal neurite outgrowth by selected optic lobe extracts. J Cell Biol 99:418a.

Carri NG, Ebendal T. 1986. A method for explantation of selected areas of the neural retina. Anat Rec 214:226–229.

Chandraker M, Agarwal S, Kriegman D. 2007. Shadow cuts: photometric stereo with shadows. IEEE Conference on Computer Vision and Pattern Recognition CVPR 2007, p 1–8.

Cruz Gaitán AM, Reynaldo M, Carri NG. 2010. Estimulación trófica de células madre embrionarias de cuerpo estriado de rata en un bioensayo 3D. In: Revista de la Asociación Colombiana de Ciencias Biológicas, Vol. E, p 43.

Dempsey WP, Fraser SE, Pantazis P. 2012. SHG nanoprobes: advancing harmonic imaging in biology. Bioessays 34:351–360.

Di Napoli J, Fiore L, Teruel LR, Carri NG, Scicolone G. 2010a. Developmental pattern of expression of epha/ephrina system in the chicken retinotectal system. In: Molecular and cellular neurobiology, p 187.

Elsdale T, Bard J. 1972. Collagen substrata for studies on cell behavior. J Cell Biol 54:626–637.

Fiore L, Di Napoli J, Alonso C, Rapacioli M, Carri NG, Scicolone G. 2010. Retinal ganglion cells axon growth depends on the grade of EphA4 phosphorylation. In: Abstracts of XII Taller Argentino de Neurociencias, Vol. 2010, p 48.

Gustafsson MG, Shao L, Carlton PM, Wang CJ, Golubovskaya IN, Cande WZ, Agard DA, Sedat JW. 2008. Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination. Biophys J 94:4957–4970.

Hamburger V, Hamilton H L. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Harrison AP. 2010. Computer vision for computer-aided microfossil identification. Master's Thesis, for Master of Science, University of Alberta, ©Adam P. Harrison, Spring 2010, Edmonton, Alberta, Canada.

Harrison AP, Wong CM, Joseph D. 2011. Virtual reflected-light microscopy. J Microsc. 244:293–304.

Haug J, Haug C, Maas A, Fayers S, Trewin N, Waloszek, D. 2009. Simple 3D images from fossil and recent micromaterial using light microscopy. J Microsc 233:93–101.

Le Baccon P. 2012. Biology and microscopy: the friendship strengthens. Bioessays 34:329.

Marubashi K, Tokumaru M, Jackson BV, Clover J M. 2011. Necessity of 2D image data for determining 3D configurations of magnetic clouds. American Geophysical Union, Fall Meeting 2011-abstract #SH21A-1911.

Ortalli AL, Fiore L, Di Napoli J, Rapacioli,M, Salierno M, Etchenique R, Flores V, Sanchez V, Carri NG, Scicolone G. 2012.

"EphA3 expressed in the chicken tectum stimulates nasal retinal ganglion cell axon growth and is required for retinotectal topographic map formation," recently published in PloS ONE and is available online at http://dx.plos.org/10.1371/journal.pone.0038566 Pasquale EB (2005) Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 6:462–475.

Pertusa Grau JF. 2003.Técnicas de análisis de imagen. Aplicaciones en Biología. Material de Educación 65, Universidad de Valencia, España.

Scicolone G, Ortalli AL, Carri NG. 2009. Key roles of Ephs and ephrins in retinotectal topographic map formation. Brain Res Bull 79:227–247.

Shao L, Kner P, Rego EH, Gustafsson MG. 2011. Super-resolution 3D microscopy of live whole cells using structured illumination. Nat Methods 8:1044–1046.

Solovei I, Cremer M. 2010. 3D-FISH on cultured cells combined with immunostaining. Methods Mol Biol 659:117–126.

Swedlow JR. 2012. Innovation in biological microscopy: current status and future directions. Bioessays 34:333–340.

Toomre D, Bewersdorf J. 2010. A new wave of cellular imaging. Annu Rev Cell Dev Biol 26:285–314.

van Teeffelen S Shaevitz JW, Gitai Z. 2012. Image analysis in fluorescence microscopy: bacterial dynamics as a case study. Bioessays 34:427–436.