

Media Cybernetics Applications Note

The Basics of Using 3D Constructor[®] 5.0

Robyn Rufner, Ahdeah Pajooohesh-Ganji and Anastas Popratiloff
Department of Anatomy and Cell Biology, The George Washington University Medical Center, Washington, DC

Introduction

The reconstruction of confocal optical sections is necessary to obtain an in-depth understanding of relationships of cellular compartments using fluorochromes. 3D Constructor 5.0, a plug-in module for Image-Pro[®] Plus v. 5.0, provides a straightforward approach for the visualization of the morphological localization and organization of labeled proteins.

Applications and Examples of Orthogonal Slices and Animation Features:

Integrin Expression in Mouse Cornea¹.

The cells that give rise to the stratified squamous epithelial cells on the cornea reside at the corneoscleral junction called the limbus. The limbal basal cells are the source of cells for the corneal epithelium and are known as the limbal stem cells (LSCs). Since the cornea is exposed to outside world, LSCs routinely generate corneal epithelial cells to maintain the corneal integrity through normal wear and tear. Of course, there are cases in which the injury is too severe to be healed via this mechanism. This is where corneal transplants and stem cell biology come into play.

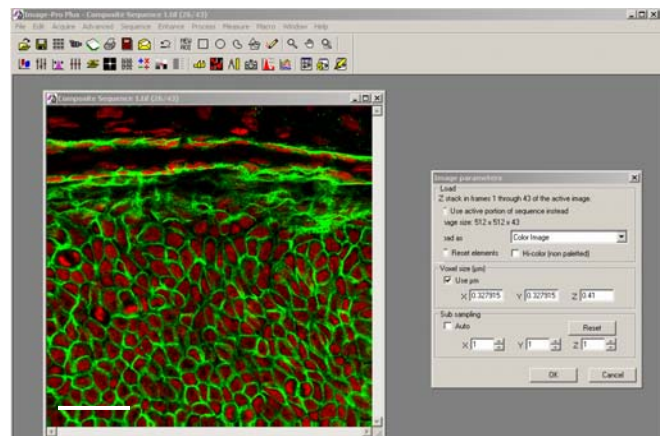
Therefore, we are trying to characterize the stem cells of the mouse corneal surface using surface markers such as integrins, which are proteins that are involved in cell adhesion. In order to do so, mouse pups are injected with 5-Bromo-2'-deoxyuridine (BrdU), which gets incorporated into DNA in place of thymidine. As cells divide, the BrdU divides as well. Analyzing the corneal surface 6 weeks after injection allows us to evaluate cells that are "label-retaining" or "slow-cycling"; a characteristic of stem cells. By characterizing the integrin expression profiles of BrdU label-retaining cells, we may be able to find markers for the LSCs, which in turn will help us to better

understand the stem cell biology associated with corneal regeneration.

Immunofluorescence was performed with whole mount preparations of mouse cornea using Alexa Fluor[®] 488 ($\alpha 3$ integrin, green) and propidium iodide (nuclear DNA, red). Forty-three 0.5 μ m laser confocal sections (acquired with a 60x oil objective, NA = 1.4) were opened in *Image-Pro Plus* 5.0 (green and red channels) \Rightarrow "Process" \Rightarrow "Color Composite" \Rightarrow add green \Rightarrow "OK" \Rightarrow add red \Rightarrow "OK" \Rightarrow "Make Sequence" \Rightarrow "Save as" (seq format). Close All Inactive Files.

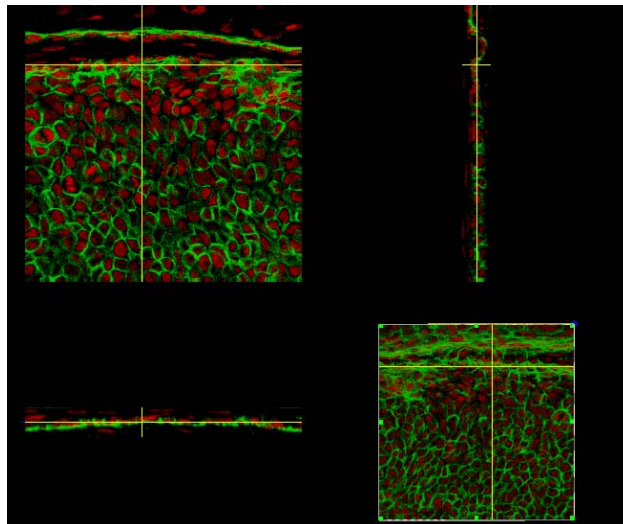
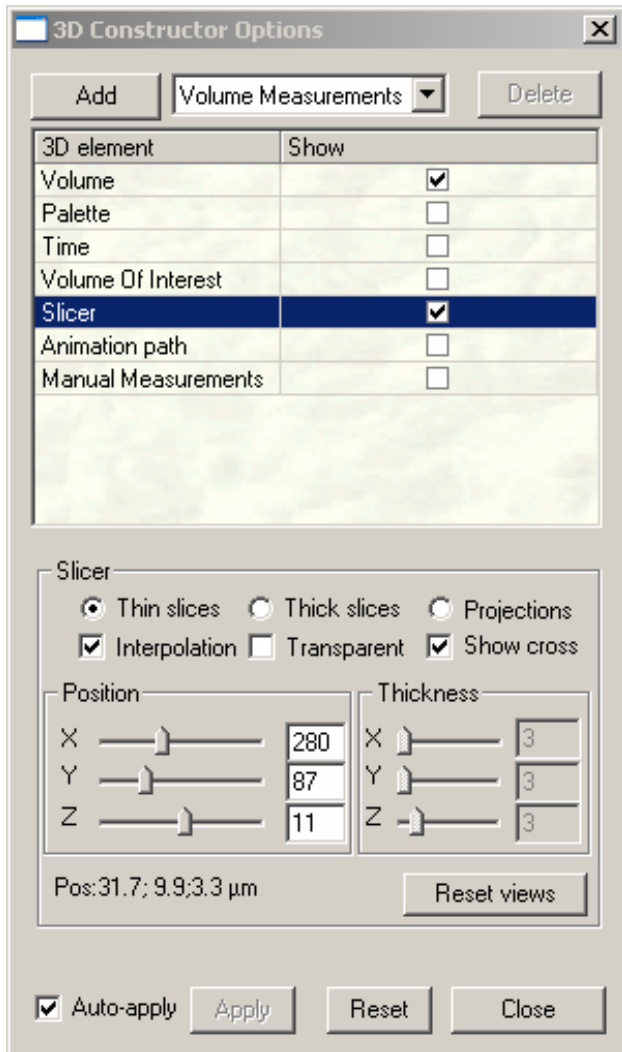
Open 3D Constructor

("Advanced" \Rightarrow "3D Constructor" or use the 3D Constructor toolbar icon). In the "Image Parameters" menu, use Z stack, microns and a sub-sampling of 1 x 1 x 1 \Rightarrow "OK". If the data set is very large, increase the sub-sampling size or use the "Auto" mode. The reconstruction of the confocal sections will appear in the "3D Constructor" menu.



3D Constructor Image Parameters Menu (Bar = 50 μ m)

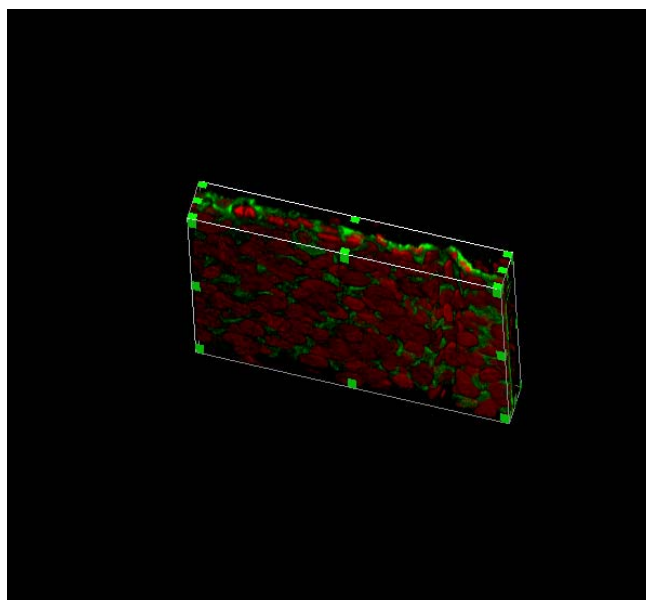
To View Orthogonal Slices, Select “Slicer” in the “3D Constructor Options” menu.



Orthogonal Slices of 43 Confocal Sections of Mouse Cornea

To Rotate the 3D Image, click on the “Hand” (right toolbar) and move the stack to the view or volume of interest (VOI).

To Change the Size and/or to Reduce the Number of Sections, select the “Arrow” (right toolbar), click on a green marker and move the arrow in the X, Y and/or Z-axis while depressing the left mouse button. If “handles” are not present, highlight “Volume Of Interest” in the “3D Constructor Options” menu. Click “Show Labels” in the “Volume of Interest” menu. Depress the keyboard “Ctrl” key to center the stack with the “Hand” tool.



Rotated Image Stack with “Handles”

To Capture an Individual View of the 3D Stack, highlight “Animation Path” and set the “Camera Position” to 0 and the “Frames per Camera Position” to 1. Click the “Folders” icon. The captured image will be located behind the “3D Constructor Options” menu. Select the new image ⇒ “File” ⇒ “Save as” (tiff format).

To Make a Movie Using the Animation Path, set the “Camera Position” to 1. Click the camera (red circle) and move the stack via the “Hand” tool as desired ($\approx 1\text{cm}$). Select the camera, repeating the above until all images have been recorded. Select the “Folders” icon. A sequence will be created of the individual views. Once completed, the new sequence will be located behind the “3D Constructor Options” menu. Select the new sequence ⇒ “File” ⇒ “Save as” (avi format).

Significance:

The use of the 3D Constructor[®] 5.0 module made it possible to visualize the localization pattern of $\alpha 3$ integrin in both the basal and the apical epithelium cells of whole mount preparations of mouse cornea. This capability has increased our understanding of limbal stem cells (LSCs) associated with current research investigations and will be instrumental with determining the direction of future studies.

¹Pajoohesh-Ganji A, S Pal-Ghosh and MA Stepp 2004. Regional distribution of $\alpha 9\beta 1$ integrin within the limbus of the mouse ocular surface. *Dev Dynamics* 230:518-528.

Applications and Examples of 3D Co-Localization:

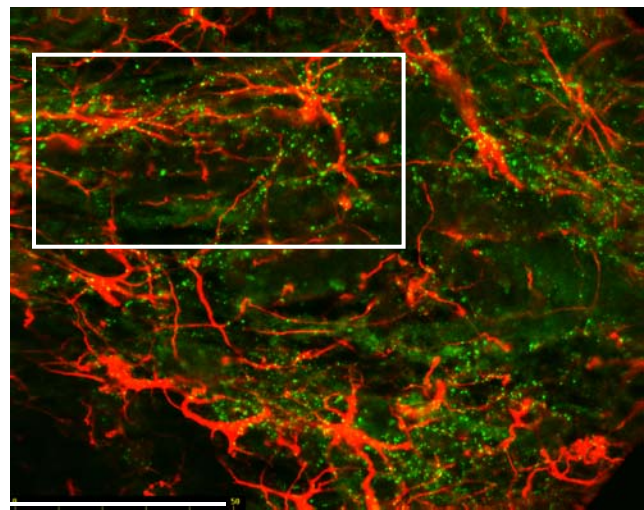
Connexin 43 in GFAP positive astrocytes². In the brain, a fraction of the astrocytic glia contains high levels of glial fibrillary acidic protein (GFAP). Specific antibodies for GFAP have been well characterized and broadly used as an astrocytic marker. Connexin 43 (Cx43) is an integral protein of some gap junctions and it is also highly expressed in the astrocytes. Gap junctions are large pores that provide communication between cytoplasm of adjacent astrocytes. Because of the vast number of gap junctions, astrocytes form a syncytium that is important for fast dissipation of ions and other small molecules. The number of gap junctions

present in astrocytes vary during development and after brain injuries. Therefore, in experimental conditions, one needs to have a reliable way of estimating and comparing the expression of Cx43 in GFAP-positive astrocytes. We use the chick tangential vestibular nucleus as a model where the expression of both Cx43 and GFAP follows a specific developmental schedule.

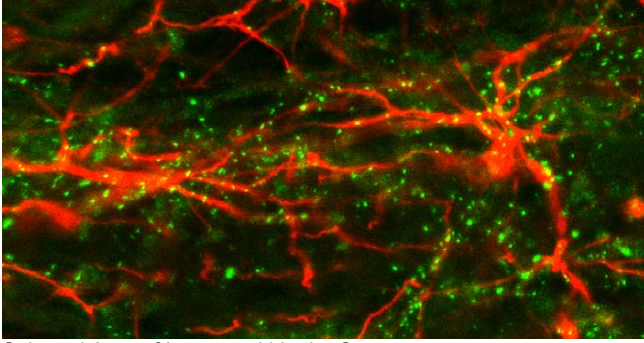
²Popratiloff A, SM Pollack, C Giaume and KD Peusner 2003. Differential expression of connexin 43 in the chick tangential vestibular nucleus. *J Neurosci Res* 71:617-628.

Immunofluorescence was performed with vibratome sections ($50\mu\text{m}$) of the tangential vestibular nucleus region of chick brain using Alexa Fluor 488[®] (Connexin 43, gap junctions, green) and Alexa Fluor 647[®] (GFAP, astrocytes, shown in red). Image stacks, consisting of 22 laser confocal sections ($0.5\mu\text{m}$) acquired with a 40x oil objective (NA = 1.35), were opened in *Image-Pro Plus* v5.0 (green and red channels) ⇒ “Process” ⇒ “Color Composite” ⇒ add green ⇒ “OK” ⇒ add red ⇒ “OK” ⇒ “Make Sequence” ⇒ “Save as” (seq format). Inactive files were closed.

Select an Area of Interest. If one is interested in a specific region, select an “AOI” ⇒ “Edit” ⇒ “Duplicate/Crop to AOI” ⇒ Save as (seq format).

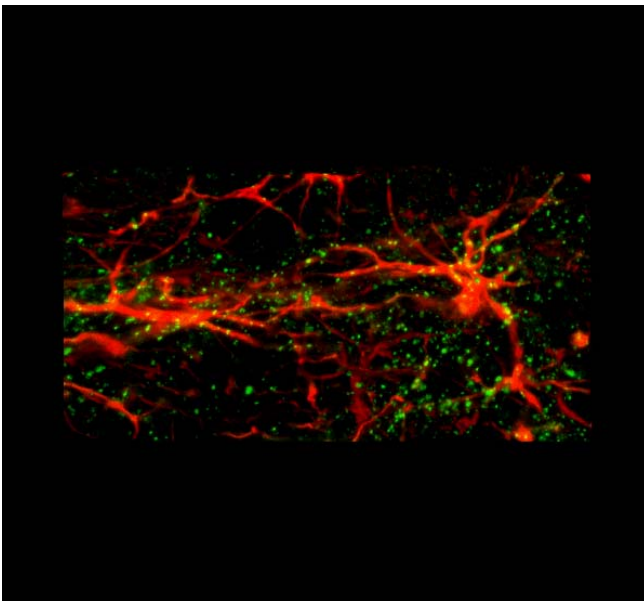


Sequence of 22 Confocal Sections of Chick Brain (Bar = $50\mu\text{m}$)



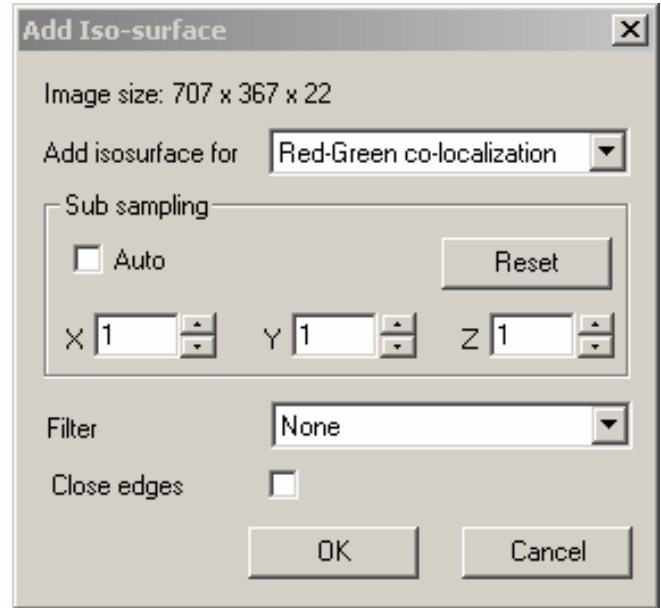
Selected Area of Interest within the Sequence

Open 3D Constructor (“Advanced” ⇒ “3D Constructor” or the 3D Constructor icon). In the “Image Parameters” menu, use the Z stack, microns and a sub-sampling of 1 x 1 x 1 ⇒ “OK”. If the data set is very large, increase the sub-sampling size or use the “Auto” mode.

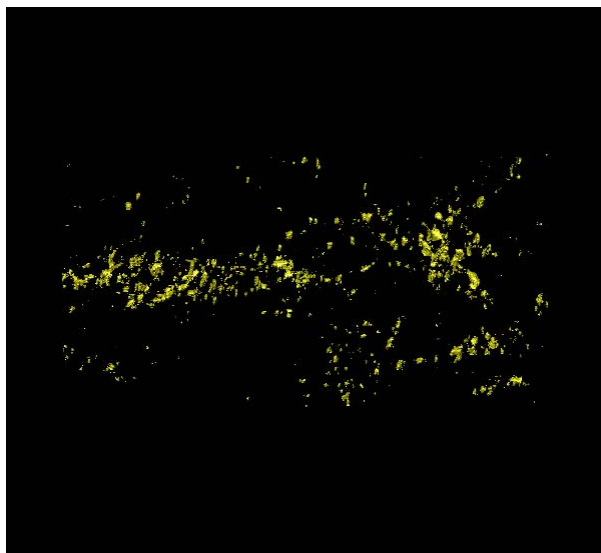
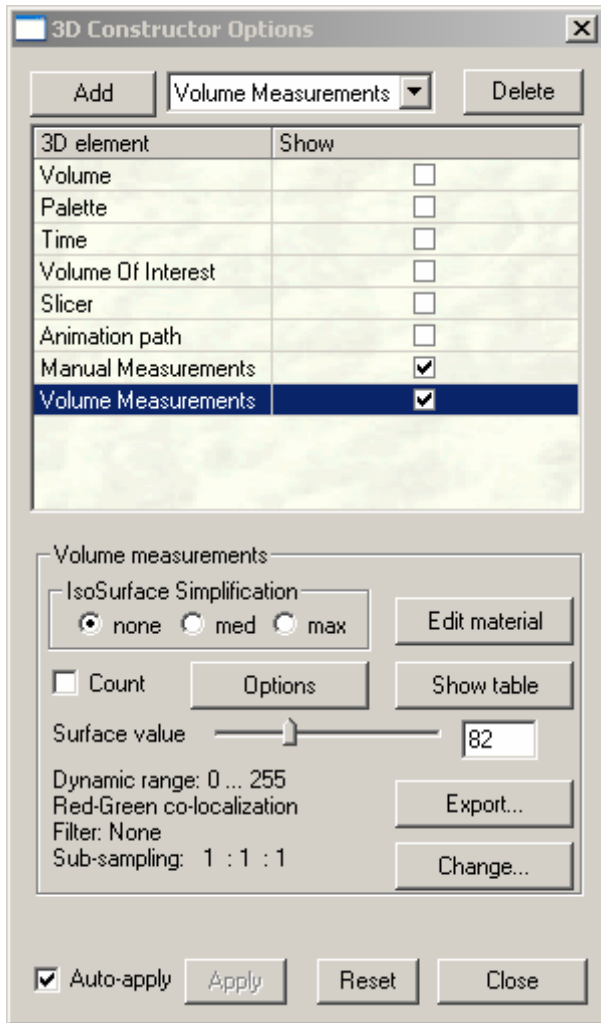


3D Constructor Sequence of the Selected Area of Interest

3D Co-Localization. In the “3D Constructor Options” menu, select “Volume Measurements” ⇒ Add ⇒ Add Iso-Surface ⇒ Red-Green co-localization ⇒ Sub-Sampling of 1 x 1 x 1 without a filter. Highlight “Volume Measurements” and insure “Volume” is not selected.



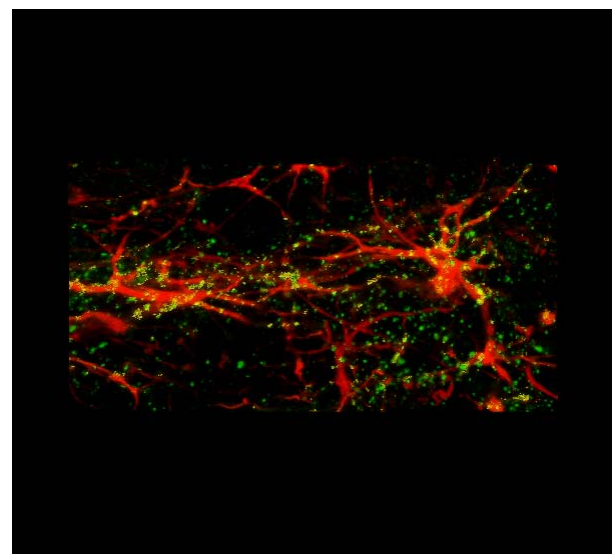
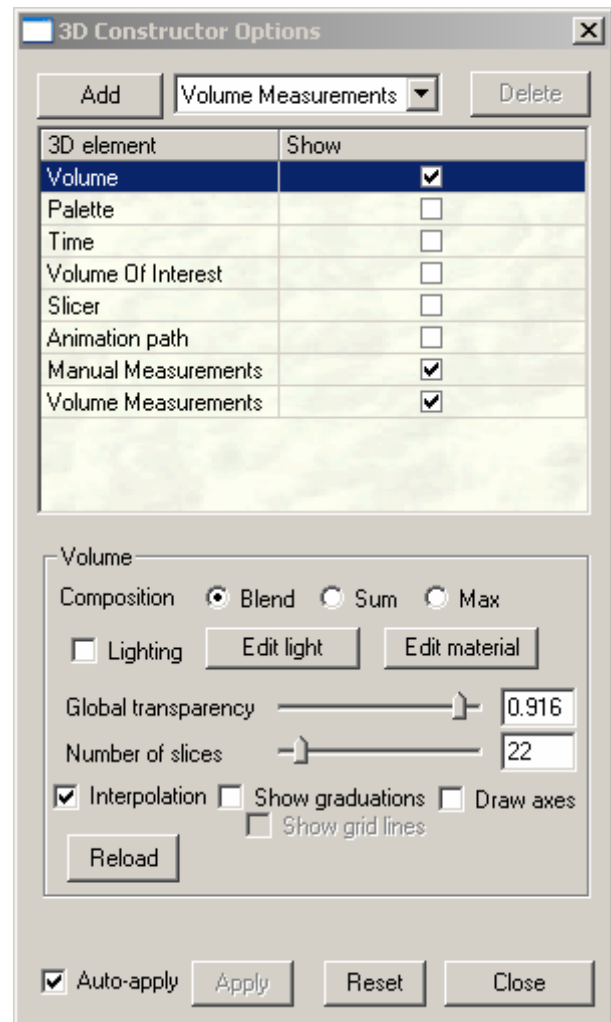
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3D Co-Localized Iso-Surface of Red-Green Pixels

To overlay the encoded co-localization iso surface with the original sequence, highlight and check “Volume”. Move the “Global

transparency” cursor until the sequence images are visible.



Global Transparency Iso-Surface Sequence

Significance:

The use of the 3D Constructor[®] 5.0 module was essential for determining the relationship of red-green pixel volume co-localization present within the astrocytes. A large fraction of Cx43 clusters was found within GFAP-positive profiles, which represented astrocytic gap junctions. In addition, the co-localization iso-surfaces revealed that some of the Cx43 labeled clusters are in close apposition to GFAP positive profiles. This suggested that Cx43 is also expressed in another cell population that interacts with GFAP-positive astrocytes via gap junctions.

Co-localization pixel coefficient values can be obtained using "Measure" ⇒ "Co-Localization" in *Image-Pro Plus* 5.0 for the red and green channels of each confocal section.

Imaging Instrumentation: A Bio-Rad MRC 1024 confocal laser scanning microscope (Hercules, CA) equipped with a krypton-argon

laser and an Olympus IX-70 inverted microscope (Melville, NY) was used to image the localization of α 3 integrin or Connexin 43 (488nm laser line excitation; 522/35 emission filter), propidium iodide (568nm excitation; 605/32 emission filter) or GFAP (647nm excitation; 680/32 emission filter). Optical sections ($Z=0.5\mu\text{m}$) of confocal epifluorescence images were acquired sequentially with Bio-Rad *LaserSharp* v3.2 software at the GWUMC Center for Microscopy and Image Analysis. <http://www.gwumc.edu/research/core/cmia/>

See Also

- Media Cybernetics Product Note- "Creating Spatial Measurements in 3D Constructor[®] 5.0"
- Media Cybernetics Product Note- "Creating Volume Measurements in 3D Constructor[®] 5.0"

Media Cybernetics, Inc.
8484 Georgia Avenue
Silver Spring, MD 20910 USA
Phone: +1-301-495-3305
Fax: +1-301-495-5964

Email: info@mediacy.com
Web: www.mediacy.com

UK: Phone: +44-(0)-1189-324324
Fax: +55-11-4427-9527

Europe: Phone: +31-715-730-639
Fax: +31-715-730-640

Asia Pacific: Phone: +65-6245-4965
Fax: +65-6245-4967

