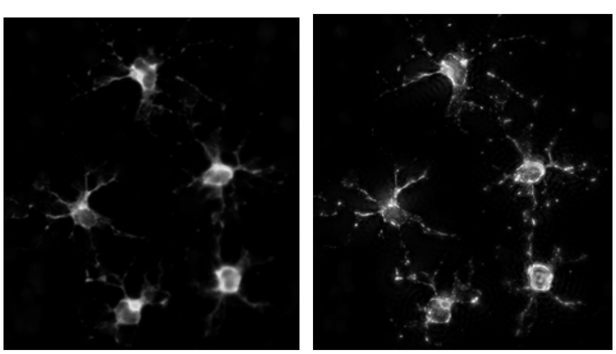
Image-Pro® Plus Product Note SharpStack®

Introduction

Two and three-dimensional fluorescent imaging is becoming an everyday occurrence in biological laboratories. The equipment needed to collect these types of images has become quite affordable and readily available. Computer technology has also advanced to the point where the ability to acquire and process single images and image stacks is both easy to handle and affordable.

A common problem encountered with two and three dimensional fluorescence imaging is the presence of light haze in the image. The haze is present as a result of out-of-focus light from structures above and below the plane of focus. Oftentimes this haze can obscure structures of interest within the image plane or volume.

Techniques exist to minimize haze and maximize the potential for revealing hidden structures. The most common technique is confocal microscopy- a well established and reproducible means for subtracting haze from images. Confocal microscopes can be expensive however, and other techniques have been developed to bring sharp, clear image sets into the realm of most research labs. One such method is deconvolution. Several methodologies exist for this particular approach. Media Cybernetics offers SharpStack, a combination of three separate routines involving commonly used and highly reproducible haze removal and image restoration techniques.



Texas Red-labeled neurons from rat spinal cord before (left) and after (right) application of SharpStack's Inverse Filter function. Images courtesy of Dr. Martin Marsala, University of California- San Diego.

Applications and Examples

SharpStack makes available one deconvolution technique (based upon the blind deconvolution concept) and two deblurring techniques. Each may be applied in a particular environment to achieve the optimum amount of image improvement.

The Inverse Filter found in SharpStack is a blind deconvolution method that seeks to restore out-of-focus haze to its point of origin within the image volume. The No Neighbor deblurring technique may be used to physically remove haze from a single image plane, while the Nearest Neighbor deblurring method may be the best method to remove approximated haze from image stacks containing fewer than five image planes. Nearest Neighbor is useful for these thinner volumes as there may not be enough information to construct a proper point spread function. But the technique itself may be used for any number of planes and is significantly faster than the inverse filter method. In each case the end result is an image with improved clarity. Use of the Inverse Filter often results in a brighter image with faint structures suddenly becoming quite visible.

SharpStack must be applied to monochrome images and image sets. However, you can acquire monochrome image sets of differing wavelengths and merge them together after using SharpStack. **SharpStack is not designed for use with image sets acquired through confocal microscopes.**

Which Method Should I Use?

Since three separate methods can be employed to improve image quality, you may want to know which method is most appropriate for a given application. The following chart can be used for making this determination.

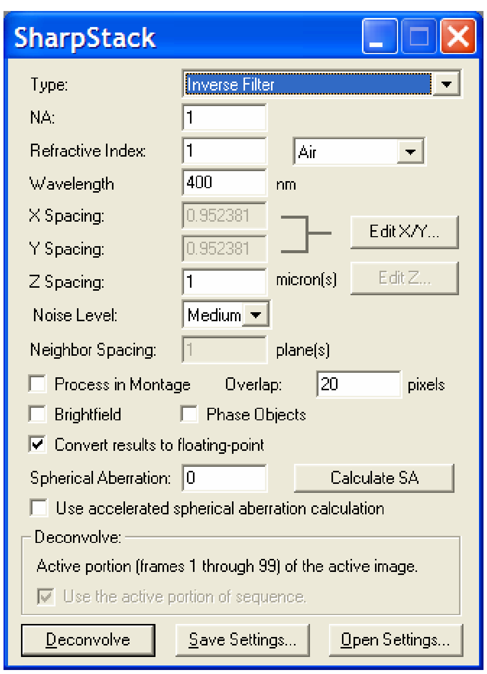
	No Neighbor	Nearest Neighbor	Inverse Filter
Fluorescence			
▪ Single Plane	•		
▪ 3-5 Planes		•	•
▪ >5 Planes			•
▪ Quantitation			•
▪ Quick Inspection	•	•	
Brightfield/Phase			
▪ Single Plane	•		
▪ 3-5 Planes		•	•
▪ >5 Planes			•
▪ Quick Inspection	•	•	

Briefly, if you are looking at a single image plane and want to remove haze, use the No Neighbor method. Images from three to five image planes in size are best cleaned up with the Nearest Neighbor method. Anything over five image planes and it's best to use the Inverse Filter. And if quantitative fluorescence is your goal, stick with the Inverse Filter as it is non-subtractive. In other words, it doesn't remove signal from the image, it reassigns photons to their points of origin. The other two techniques subtract haze from the image to clarify structure.

Deconvolved images may be further enhanced with other Media Cybernetics modules such as 3D rendering with 3D Constructor® or with Image-Pro Plus techniques such as Color Composite or Extended Depth of Field. In-depth product notes for these modules and commands can be found on our web site- www.mediacy.com. Click the 'Applications' link and you'll find the descriptions under 'Product Notes'.

Implementation

Advanced|SharpStack…



The SharpStack Interface

Type: This drop-down list contains deblurring and deconvolution methods. The options in this drop-down list change in SharpStack to reflect the type of deblurring or deconvolution that you have selected.

Three methods are available- No Neighbor (Fig.1), Nearest Neighbor (Fig. 2) and Inverse Filter (Fig. 5). Each of the options for the enhancement method you select is discussed in the appropriate section.

No Neighbor/Nearest Neighbor

- Click the down arrow to select an image enhancement method.
- Click 'No Neighbor'.

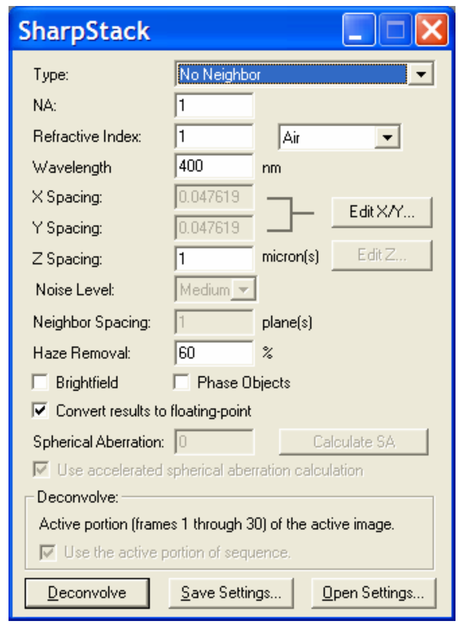


Fig. 1. The No Neighbor Interface

- Click the down arrow to select an image enhancement method.
- Click 'Nearest Neighbor'.

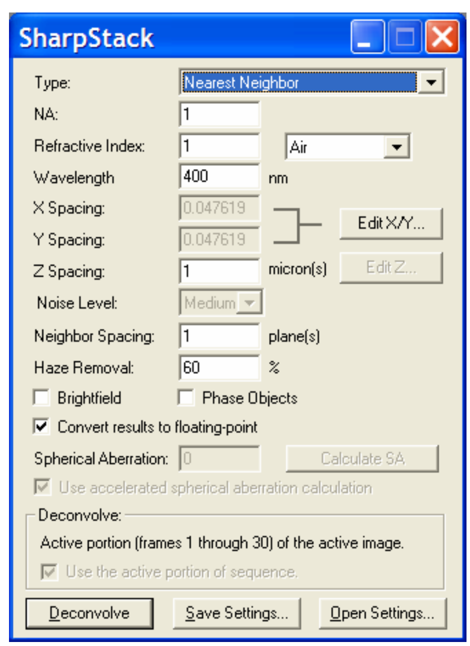


Fig. 2. The Nearest Neighbor Interface

NA- In this box, type the numerical aperture for the objective lens used to acquire the image.

Refractive Index- Indicates the immersion media used to couple the objective lens to the specimen. Five choices are available; Air, Water, Oil, Glycerin and Custom. It is very important to select the appropriate immersion media!

When you have selected the immersion medium, the correct refractive index for that medium will be updated in the adjacent refractive index box.

Wavelength- Type the value for the peak wavelength of the fluorochrome you are acquiring. It is very important to indicate the peak wavelength in nanometers!

X Spacing- The calibrated horizontal distance between pixels in the CCD imager.

Y Spacing- The calibrated vertical distance between pixels in the CCD imager.

- If the CCD imager has been calibrated to the objective lens used to acquire the image, the 'Edit…' button will allow you to select the appropriate spatial calibration file. Upon selecting the file, the X and Y Spacing options will be automatically updated with the appropriate dimensions. Spatial calibrations must be calibrated in microns!

Z Spacing- The distance between Z planes in an image stack. If you use the AFA™ Plug-In module to acquire the image, the value in this field will be automatically updated. If images were acquired in an environment other than Image-Pro Plus or Discovery, the spacing values will also be automatically updated. It is very important to indicate the correct Z spacing!

Neighbor Spacing- Applies a 'smoothing' factor to remove noise from images. A value between 1 and 5 may be entered, with the default being 1. The larger the value, the more noise removal will occur. The Neighbor Spacing option is only available with the Nearest Neighbor deblurring method.

Caution- Be careful when applying a larger value, as it may remove signal and structures of interest from the resulting image.

Haze Removal- This value indicates the percentage of haze you would like to remove from the image. Use a value between 50-65% to start. See Figure 3 for the results of using different haze removal percentages.

Caution- Values higher than 50-65% can remove potentially important information from the image.

Brightfield- Click this checkbox if you are applying the algorithm to a brightfield image.

Phase Objects- Click this checkbox if you are applying the algorithm to an image that has been created in a phase contrast type environment, for example images created by phase contrast optics, Nomarski, reducing the condenser aperture, etc.

Note- It is not necessary to check the brightfield box for phase objects.

Convert results to floating point- Converts the resulting data into the floating point format, which creates a nicer looking image. Technically, this means the resulting data are left in the floating point format that is used for internal calculations. This prevents clipping that is inherent in converting floating point data to integer data, and often produces a better looking image. For the No Neighbor and Nearest Neighbor methods of deblurring, the option to display data in the floating point format is automatically selected, and the option is grayed out in the interface.

Deconvolve:- This grouping allows you to apply the selected algorithm to the whole image stack or a subset of image planes.

Note- if an AOI is applied to the image stack, only the AOI will be deconvolved.

- Click the checkbox next to "Use active portion of the active image" if you have made adjustments to the image stack through the use of the Sequence Toolbar (Acquire|Sequence Tools|Toolbar…)

'Deconvolve'- Starts the selected deblurring/deconvolution algorithm.

‘Save Settings…’- Saves the current settings for use with similar images in the future. These settings are saved with the file extension .dcs.

‘Open Settings…’- Recalls saved .dcs settings for use with an image.

No/Nearest Neighbor Helpful Hints

- Make sure you have specified the correct NA, Refractive Index, Peak Emission Wavelength and x, y and z Spacing.
- Set Haze Removal to 50% to start. Otherwise you may remove something you want to see!
- Use Neighbor Spacing to remove noise from images being deblurred with the Nearest Neighbor technique.
- If it’s necessary make sure you indicate whether your sample is brightfield or phase contrast
- Convert your results to floating point images.

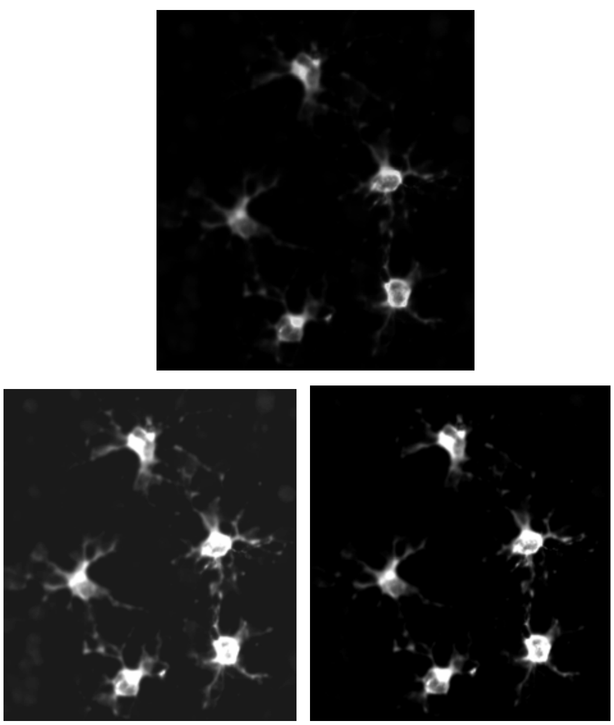


Fig. 3. Application of No Neighbor filter to single image plane. Raw image (top), with 50% haze removal (lower left) and 95% haze removal (lower right). Notice the lack of structure present in the image as the percentage of haze removal increases.

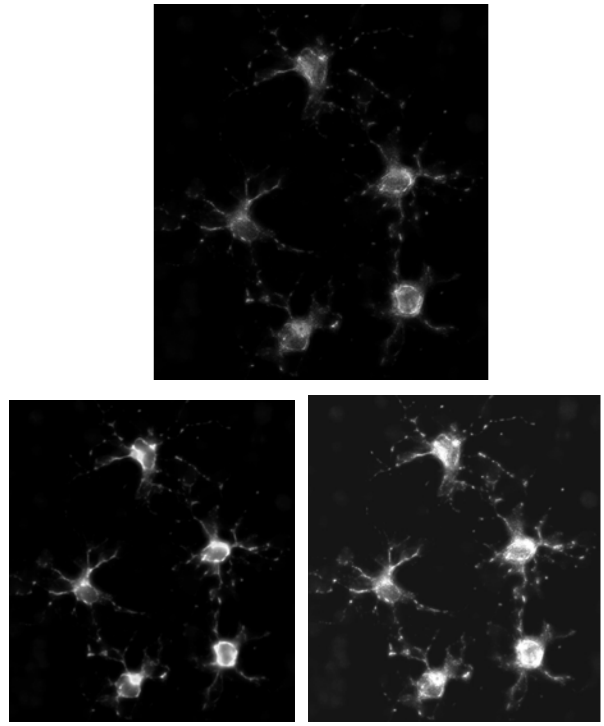


Fig. 4. Application of Nearest Neighbor filter to image 12-plane image volume. Raw image stack (top), with 50% haze removal (lower left) and 95% haze removal (lower right). Although the lower right image appears at first pass to be more resolved, more detail has been omitted and image data appears to be noisier.

Inverse Filter

- Click the down arrow to select an image enhancement method.
- Click ‘Inverse Filter’.

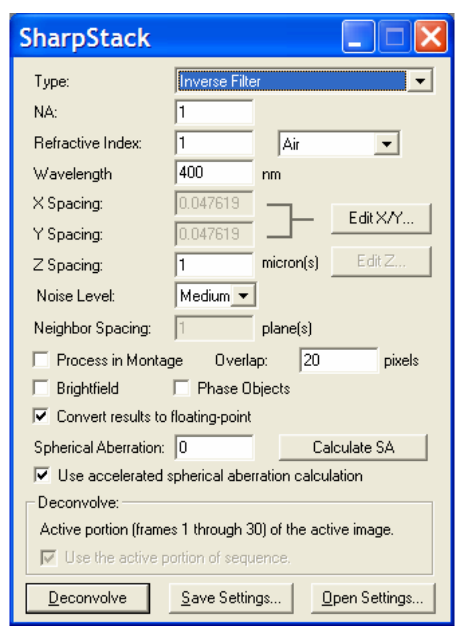


Fig. 5. The Inverse Filter Interface

NA- In this box, type the numerical aperture for the objective lens used to acquire the image.

Refractive Index- Indicates the immersion media used to couple the objective lens to the specimen. Five choices are available; Air, Water, Oil, Glycerin and Custom. It is very important to select the appropriate immersion media!

When you have selected the immersion medium, the correct refractive index for that medium will be updated in the adjacent refractive index box.

Wavelength- Type the value for the peak wavelength of the fluorochrome you are acquiring. It is very important to indicate the peak wavelength!

X Spacing- The calibrated horizontal distance between pixels in the CCD imager.

Y Spacing- The calibrated vertical distance between pixels in the CCD imager.

- If the CCD imager has been calibrated to the objective lens used to acquire the image, the 'Edit…' button will allow you to select the appropriate spatial calibration file. Upon selecting the file, the X and Y Spacing options will be automatically updated with the appropriate dimensions. Spatial calibrations must be calibrated in microns!

Z Spacing- The distance between z planes in an image stack. If you use the AFA Plug-In module to acquire the image, the value in this field will be automatically updated. If images were acquired in an environment other than Image-Pro Plus or Discovery, the spacing values will also be automatically updated. It is very important to indicate the correct Z spacing!

Neighbor Spacing- Applies a 'smoothing' factor to remove noise from images. Changing this factor is not possible when using the Inverse Filter.

Process in Montage- This function breaks the image volume into smaller pieces to be separately deconvolved and then later reassembled into the complete volume. It is used with larger image volumes to overcome limitations in the manner in which Windows® treats RAM.[1]

Overlap- After images are processed in montage, the resulting blocks must be reassembled into the final image volume. Selecting an overlap value (in pixels) allows the blocks to be positioned in such a way that removes or reduces the potential for image artifacts to appear.

Brightfield- Click this checkbox if you are applying the algorithm to a brightfield image.

Phase Objects- Click this checkbox if you are applying the algorithm to a phase contrast image.

Note- It is not necessary to check the brightfield box on for phase objects.

Convert results to floating point- Converts the resulting data into the floating point format, which creates a nicer looking image.

Spherical Aberration- Allows you to input a value between -15 and 15 to correct for spherical aberration in the sample. Sources of spherical aberration include mismatched mounting/immersion media, incorrect coverslip thickness and mismatched camera couplers. The resulting images can appear blurred and lack a combination of focus, resolution and contrast.

The numerical values approximate the spherical aberration within the sample. A sample with no spherical aberration would have a value of 0, while samples with spherical aberration increasing as you go deeper into the sample would have more negative values. Samples with spherical aberration becoming more pronounced toward the top of the sample would have a more positive value.

Calculate SA- Click this button to have SharpStack automatically calculate the spherical aberration constant. A value will be returned which can then be applied to the sample through the use of Inverse

[1] Although image volumes may appear to fit easily into the amount of available RAM, the Inverse Filter requires up to five times the amount of memory for the size of the image volume being processed. This is because several virtual image volumes are created during image stack processing. Windows does not allow the use of large contiguous blocks of memory, which necessitates the Process in Montage option.

Filter. Applying an AOI to a portion of the stack will confine the calculations to the AOI and result in a quicker estimate of the appropriate correction factor to use in deconvolving the entire stack.

Use accelerated spherical aberration correction- Click this check box if you want to speed up the calculation time for spherical aberration correction. The calculation used for this method is not as stringent as the complete Spherical Aberration correction, but has no discernible effect on the outcome of the image.

Noise Level- This drop-down allows you to characterize the noise level in the sample and suppress it. Four levels are present- Auto, Low, Medium and High. The default level is Medium. Select a level that represents the noise characteristics of your image volume.

Deconvolve:- This grouping allows you to apply the Inverse Filter algorithm to the whole image stack or a subset of image planes.

- Click the checkbox next to "Use active portion of the active image" if you have made adjustments to the image stack through the use of the Sequence Toolbar (Acquire|Sequence Tools|Toolbar…)

'Deconvolve'- Starts the No Neighbor or Nearest Neighbor deblurring algorithm.
'Save Settings…'- Saves the current settings for use with similar images in the future. These settings are saved with the file extension .dcs.

See Also

Media Cybernetics Product Note- "Color Composite".
Media Cybernetics Product Note- "Extended Depth of Field".
Media Cybernetics White Paper- "Extended Depth of Field".
Media Cybernetics Solution #1275- 3D Colocalization.

Product Notes, Solution Notes and White Papers may be found on our website- www.mediacy.com and by clicking 'Applications'.

'Open Settings…'- Recalls saved .dcs settings for use with an image.

Inverse Filter Helpful Hints

- Make sure you have specified the correct NA, Refractive Index, Peak Emission Wavelength and x, y and z Spacing.
- Make sure you indicate whether your sample is brightfield or phase contrast
- Convert your results to floating point images. They will look great.
- Use Process in Montage for larger image volumes.
- Use a combination of spherical aberration correction and noise filters to improve the quality of the resulting image.

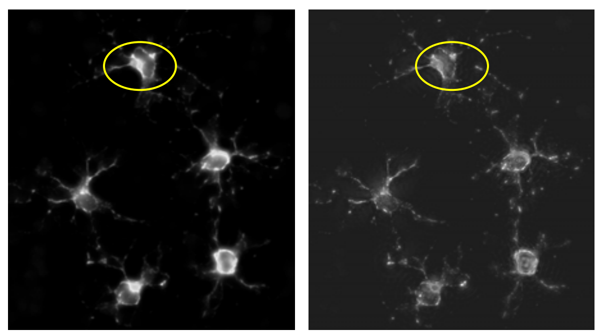


Fig. 6. Texas Red-labeled neurons from rat spinal cord. Raw image volume before (left) and after (right) application of the Inverse Filter. Contrast adjustment after filtering reveals more highly resolved central structures and dendrites. The circled neuron in the filtered image shows a higher degree of resolution than in the unfiltered image. Image courtesy of Dr. Martin Marsala, University of California- San Diego.

If You Require a More Powerful Algorithm

The Inverse Filter is quite useful for a number of different sample types. However, there may be cases where you need something a little more powerful or specialized to achieve your resolution requirements. We do have more powerful and specialized algorithms available to perform more robust deconvolution routines. Please contact your Media Cybernetics Sales Representative for more details.

Related Products

3D Constructor- Three Dimensional Display and Rendering Plug-In.
Advanced Fluorescence Acquisition (AFA)- Microscope and Peripheral Automation.
Scope-Pro® Plug-In.

How To Order

For more information on Image-Pro Plus, and to locate a Media Cybernetics' reseller in your area, visit our website at www.mediacy.com.

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