7 *HazeBuster*™

HazeBuster mathematically calculates and removes out-of-focus haze from microscope images. It uses the No Neighbor (single image) and the Nearest Neighbor (three image) algorithms. If you want to use a Constrained Iterative algorithm, please use the commands that come with **MicroTome** or **MicroTome CI**.

HazeBuster is also sold as part of the full **MicroTome** package. If you have **MicroTome**, please read this section for a description of the **Rapid Deconvolution** command. **HazeBuster** has also been called **Power HazeBuster**.

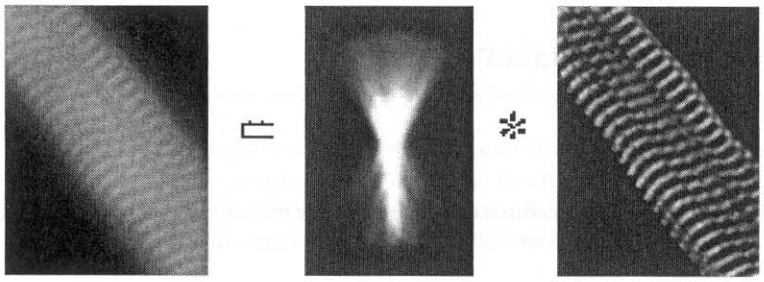
Please look in the **3D** menu to find and use the **HazeBuster** commands (as well as any other **MicroTome** commands).

7.1 Overview of Digital Deconvolution

It may be helpful to review a few basic principles of optics in order to understand how **HazeBuster** works.

7.1.1 Removing the Haze

A microscopy image includes in-focus and out-of-focus light. The out-of-focus light comes from above and below the plane of focus. The smear or blur produced by the out-of-focus planes is a natural consequence of the optics of the microscope. The out-of-focus haze is added to the image in a very precise manner by the point spread function (PSF) of the microscope. A point spread function is a mathematical term that describes how a point of light spreads out as it passes through the microscope.



Raw Image
Acquired by Camera

PSF

Object Image
Before convolution
by microscope

The Raw Image is the Product of the Convolution of the Object Image with the PSF
(* indicates convolution)

HazeBuster uses a theoretical or calculated point spread function. The theoretical PSF is calculated using diffraction theory. The parameters needed to calculate a PSF are:

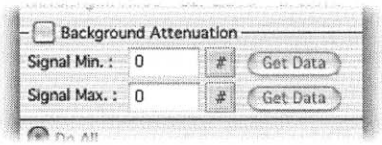
- Light wavelength
- Numerical aperture of the lens
- Refractive index of the immersion oil
- Pixel dimension within a plane and between planes (*dx*, *dy*, and *dz*)

HazeBuster can deconvolve images from a standard fluorescence microscope or a confocal microscope. Images from brightfield microscopes can also be deconvolved, but the results are less predictable.

7.1.2 Background Attenuation, or Scaling the Data

For the best possible deconvolved images, your data should span the full range of values available from your camera. For example, the data should range from 0 to 4095 for 12-bit images and from 0 to 255 for 8-bit images. If your images do not cover the full dynamic range, you can use the **Background Attenuation** option to scale the data. This step is not essential, but it can improve the signal-to-noise ratio in your data set.

Background Attenuation scales the original data before deconvolution. It alters the data in memory and leaves the original image window untouched.



Background Attenuation Section of
the **Constrained Iterative** Commands

To scale your data when using the **Constrained Iterative Frame** and **Volume** commands, set the **Data Size** pop-up menu and check the **Background Attenuation** checkbox. All values between **Signal Minimum** and **Signal Maximum** will be scaled to the image's data range.

For fluorescence images, you can remove background by raising the **Signal Minimum**. If, for example, you know that most of your background is dimmer than 200, you can set **Signal Minimum** to 800. When **MicroTome** scales the data, the background values below 200 will be excluded from the image.

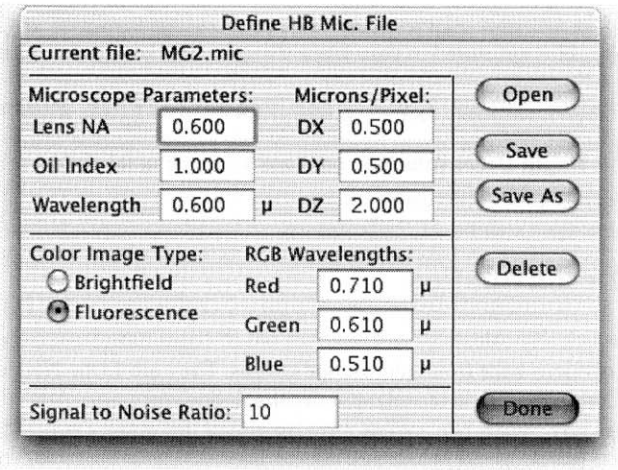
For bright-field images, you can reduce the bright background by lowering the **Signal Maximum**.

7.2 Using HazeBuster

7.2.1 Define Mic. File

The first thing you should do when using **HazeBuster** is to describe the microscope and acquisition parameters. These will be stored in the Mic. file, which **HazeBuster** will use to deconvolve your image.

You can find the **Define Mic. File** command in the **3D** menu.



Define Mic. File Dialog Box

Microscope Parameters:

Lens NA: Enter the numerical aperture of the objective. You can find this printed on the objective's side.

Oil Index: Enter the index of refraction for the medium between your objective and your sample. This number is printed on bottles of microscope oil. Oil's index is usually around 1.5. For air, you can use 1.0. For water, you can use 1.3.

Wavelength: Enter the light's wavelength in microns. This is the emission wavelength, the wavelength of the light being deconvolved. Use 0.570 for white light.

Microns / Pixel:

DX, DY: Enter the distance in microns between pixels.

You will need to measure DX and DY for each objective and each camera you use. (The image's bin size will affect this, too.) To measure DX and DY, capture an image of a stage micrometer or an object of known size. Divide the known distance by the number of pixels used to display it. Most cameras have square pixels.

DZ: Enter the distance in microns between image planes. This is the same as the step size used by the focus motor when you acquired the data.

If you are deconvolving a single image, set **DZ** to 1.0.

Color Image Type, RGB Wavelengths: **Rapid Deconvolution** uses this information for removing haze from color images.

Signal to Noise Ratio:

Increase the range of intensities within your deconvolved data by raising the SNR. You can set the ratio between 1 and 30, inclusive.

Open, Save, Save As:

Mic. files get stored in your home directory, in the IPLab Preferences folder:

`Users: YourUserName: IPLab 3.9.4 User Folder: IPLab Preferences: Mic Files`

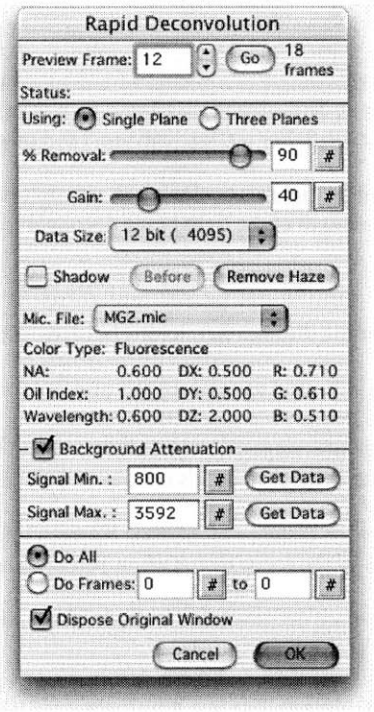
The **Rapid Deconvolution** command will look in this folder for all Mic. files, so make sure to save your Mic. files here.

7.2.2 Rapid Deconvolution

Rapid Deconvolution mathematically calculates and removes out-of-focus haze from microscope images. It has two algorithms for removing out-of-focus haze from microscope images. They are 1) the No Neighbor (single image) technique and 2) the Nearest Neighbor (three image rapid deconvolution) technique.

You must open the image to be deconvolved before you choose this command. The image can be a single frame or a sequence. **Rapid Deconvolution** can work with Byte, Short, and Unsigned grayscale images. It can also work with Color 24 and Color 48 RGB images.

Next, pick the **Rapid Deconvolution** command from the **3D** menu.



Rapid Deconvolution Dialog Box

Preview Frame: Previewing allows you to see the effects of your settings before you make them permanent. Use these tools to select which frame of the sequence to preview. Click on the up/down arrows or type in a new number and then click on the **Go** button.

Using Single/Three: Select **Single Plane** to use the No Neighbor algorithm; select **Three Planes** to use the Nearest Neighbor algorithm.

% Removal: The **% Removal** slider sets the percentage of haze removed from the image. This setting is similar to opening and closing the pinhole on a confocal microscope.

Gain: The **Gain** setting controls the image brightness during deconvolution. Usually you will adjust the **Gain** setting to make the deconvolved image about as bright as the original image.

Data Size: Set the **Data Size** to tell the software the range of intensities that the camera is capable of acquiring. Common digital cameras produce 12-bit data, with data values ranging from 0 to 4095. The **Background Attenuation** option uses the **Data Size** to scale the data.

Shadow: Checking the **Shadow** box adds a shadow function during deconvolution. The effects are similar to a DIC image.

Before / After: This button switches between the 'before deconvolution' and the 'after deconvolution' images. The button's name will toggle between **Before** and **After**. You can compare before and after images by repeatedly clicking on this button.

Remove Haze: The **Remove Haze** button will perform the deconvolution on the currently active frame and show you the results in the preview image.

Mic. File: Pick a settings file from the **Mic. File** pop-up menu.

If you want to create a new Mic file or change the settings, then you need to **Cancel** and select **Define Mic. File** from the **3D** menu (please see page 117).

Background Attenuation: Check this box to scale the data before deconvolving it. All data between **Signal Minimum** and **Signal Maximum** will be scaled to the **Data Size** range.

You can exclude fluorescence background by raising the **Signal Minimum** above the level of the background. Please see page 116 for more information about the **Background Attenuation** feature.

Get Data: Click the **Get Data** buttons to get the minimum and maximum intensities present in your image sequence.

Do All / Do Frames: Use the **Do All** or **Do Frames** options to pick which frames of an image sequence to deconvolve.

Dispose Original Window: Check this box to close the original image after deconvolution is complete.
Note: This will not save any changes you made to the original image!

OK: The **OK** button begins the deconvolution. Use this button after you have optimized the settings for the preview frame.

Cancel: The **Cancel** button will close the dialog box without performing any deconvolution and without making any changes.

The # / Var buttons let you use values stored in variables instead of normal numbers. Set the button to # to enter a regular number. Set the button to Var to enter a variable's index number; the program will then use the variable's value for that parameter.

7.3 Troubleshooting: Image Acquisition Issues

Please see the image acquisition solutions in the **MicroTome** chapter starting on page 121.

7.4 Bibliography

Please see the **MicroTome** bibliography on page 123.