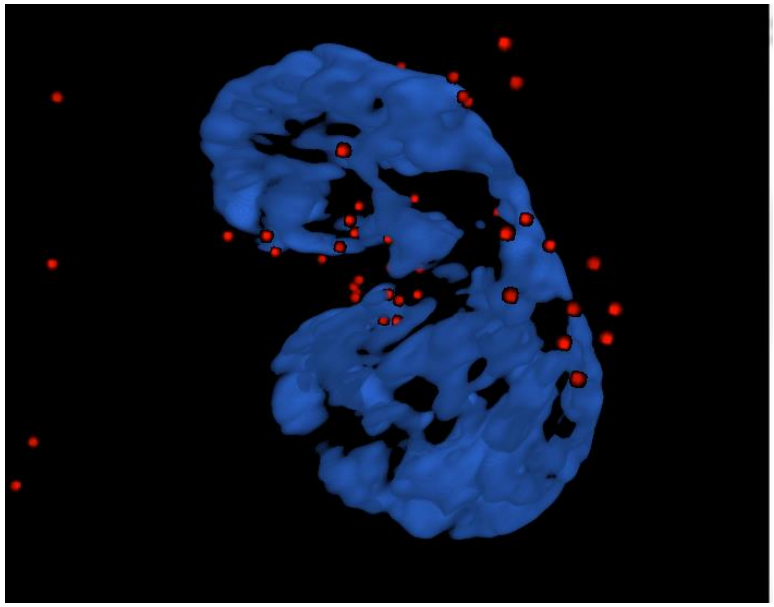
CytoViva® 3-D

The CytoViva 3-D System allows the user can locate objects of interest in a 3-D space. It does this by acquiring multiple Z planes and performing our custom software routines to locate and observe the positions of these objects. For example the location of nanoparticles can be located in relative position to a cell that is either stained or unstained.

Here red spheres show the location of gold nanoparticles inside/around a cell nucleus.



System Specifications:

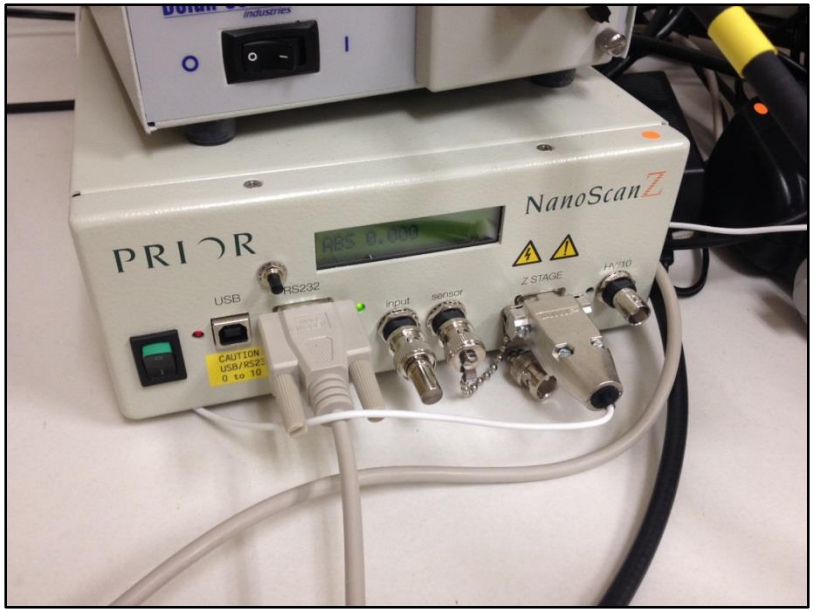
- Piezo Z-Stage System
- 14 bit Monochrome camera
- 400 micron total travel distance
- 1 Nanometer level repeatability
- 100nm step sizes
- Customized easy to use acquisition software
- 3-D software analysis that includes:
    - Deconvolution routines
    - PSF Generation
    - Numerical aperture calculation
    - Nanoparticle locator
    - 3-D viewer

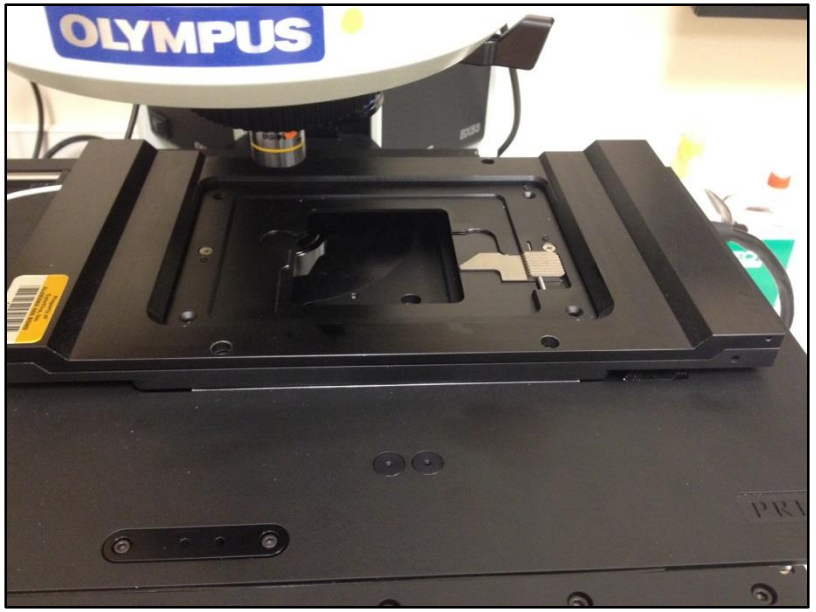
Camera:

- Q Imaging Exi Blue Monochrome
- 1392 x 1040 pixels
- Cooled down to 0˚C
- 6.45µm x 6.45µm pixel size
- High sensitivity (especially in the near IR)
- Front-illuminated interline CCD
- Binning up to 8x8
- 800Mb/s bandwidth capacity
- 15 frames per second full resolution @ 14 bits (30MHz)

Stage:

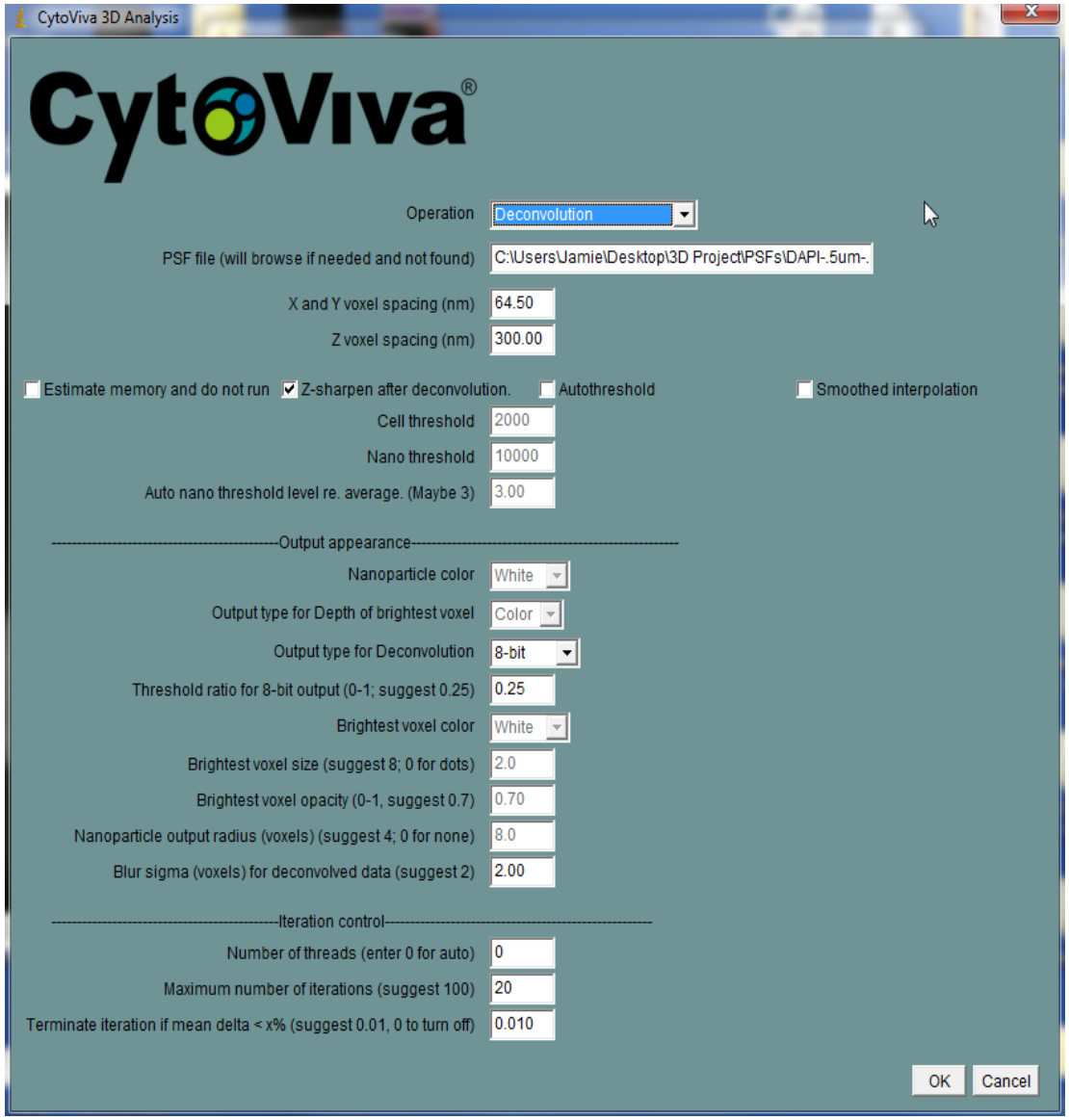
- Prior NanoScan Z
- 400µm Stage Travel Range.
- Best repeatability of 1 Nanometer
- Accuracy/Linearity of 0.5% of Travel
- Maximum Load of 500 Grams (Contact prior for larger loads)
- Stage Control via Analog (0-10 VDC) USB and RS232
- Output-Position Signal 0.0-10.0V





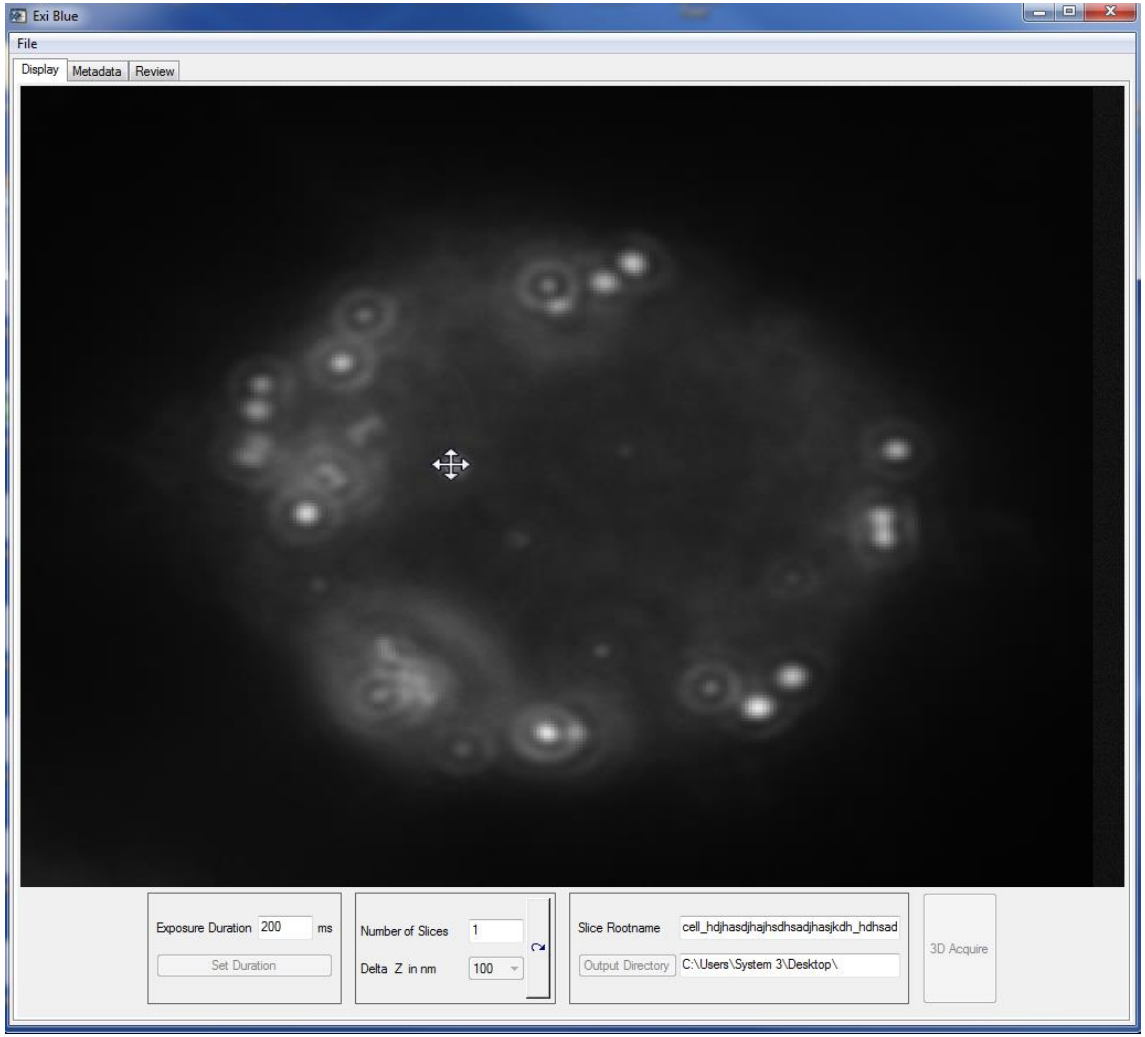
Analysis Software:

- Deconvolution routines
- PSF generation
- Nanoparticle locator
- Numerical Aperture calculator
- Interpolation routine



Acquisition Software:

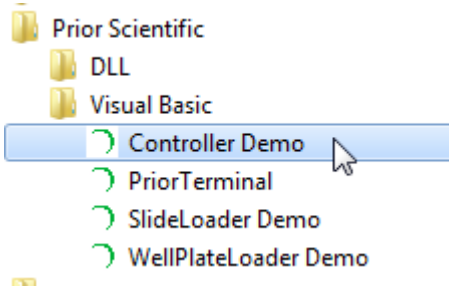
- Controls synchronization of Stage and camera
- Outputs metadata
- Control of slice spacing and number of stacks
- Review the stack
- Set exposure time



Hooking up the Nanoscan Z:

- The Nanoscan Z controller can daisy chain with the Prior Proscan controller via the RS232 connector.

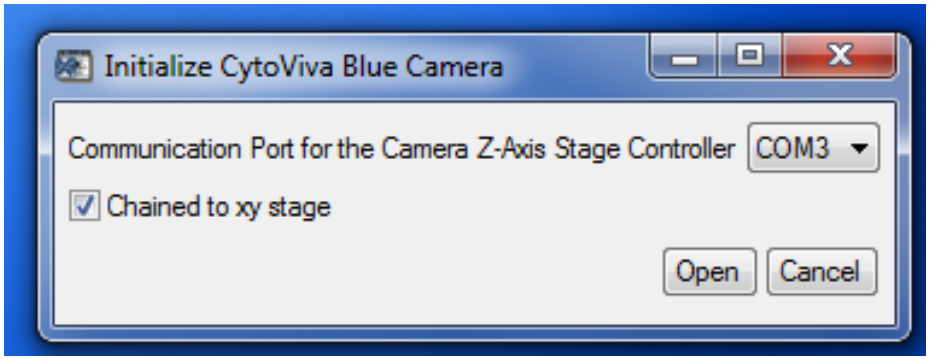
- The actual Nanoscan piezo stage mounts into the Prior XY stage via 4 screws and connects to the front of the NanoScan Z controller.
- Once all connections are made the Naoscan Z and Proscan can be powered on.
- The Prior Controller demo can also be ran to test that both stages are working.



- Once both stages are registering with the controller demo you may start the Cytoviva_3D_Imaging program on the desktop to start the Acquisition Software.

Acquiring a stack:

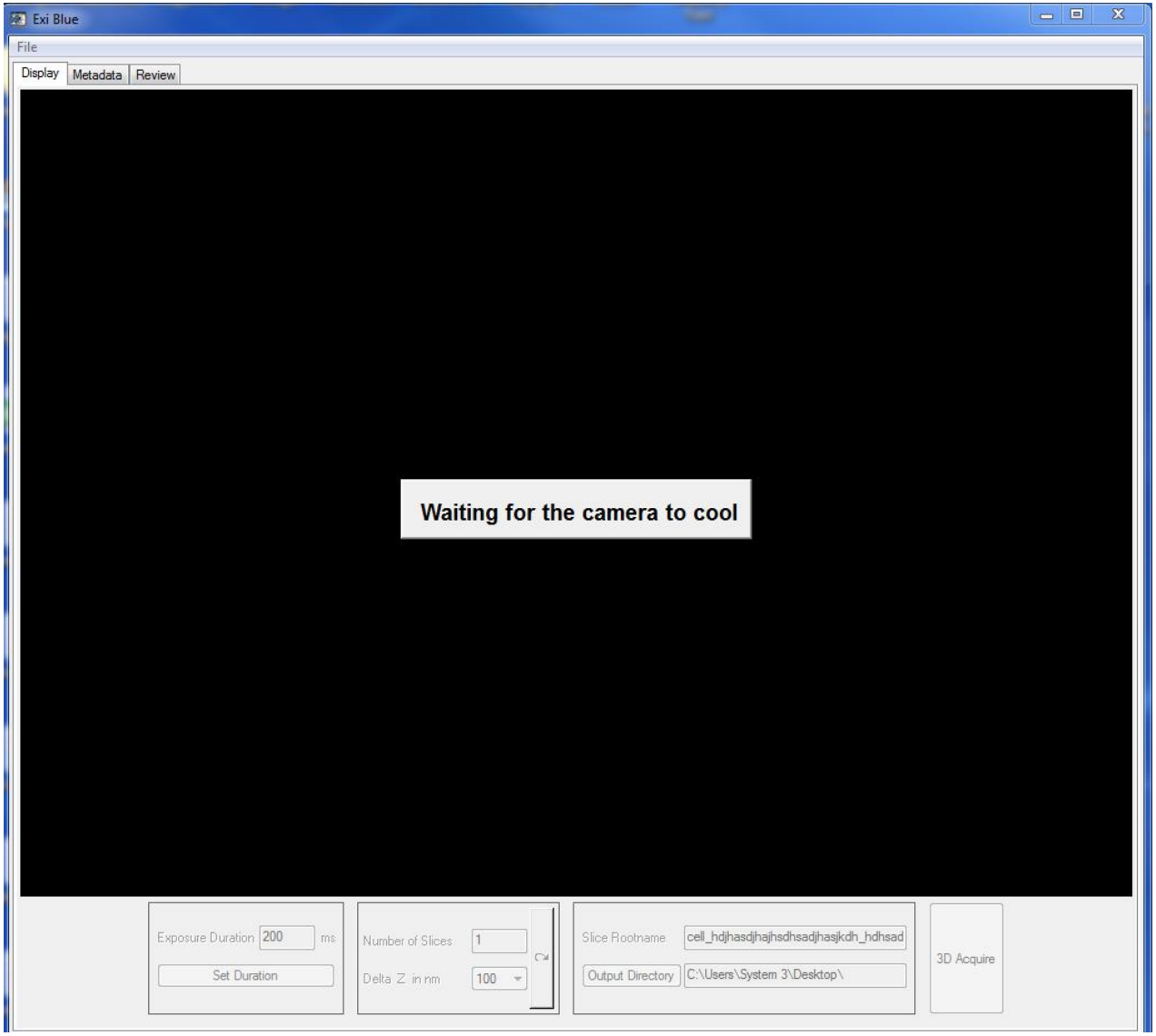
1. Place slide on stage and image as normal
2. For best results for cells and tissue use 60x or 100x magnification.
3. On the desktop click on the “Cytoviva_3D_Imaging”icon.
4. The “Initialize CytoViva Blue Camera Z-Axis Stage Controller” pop-up window appears. Make sure the “Chained to xy stage” box is checked and COM3 is selected.



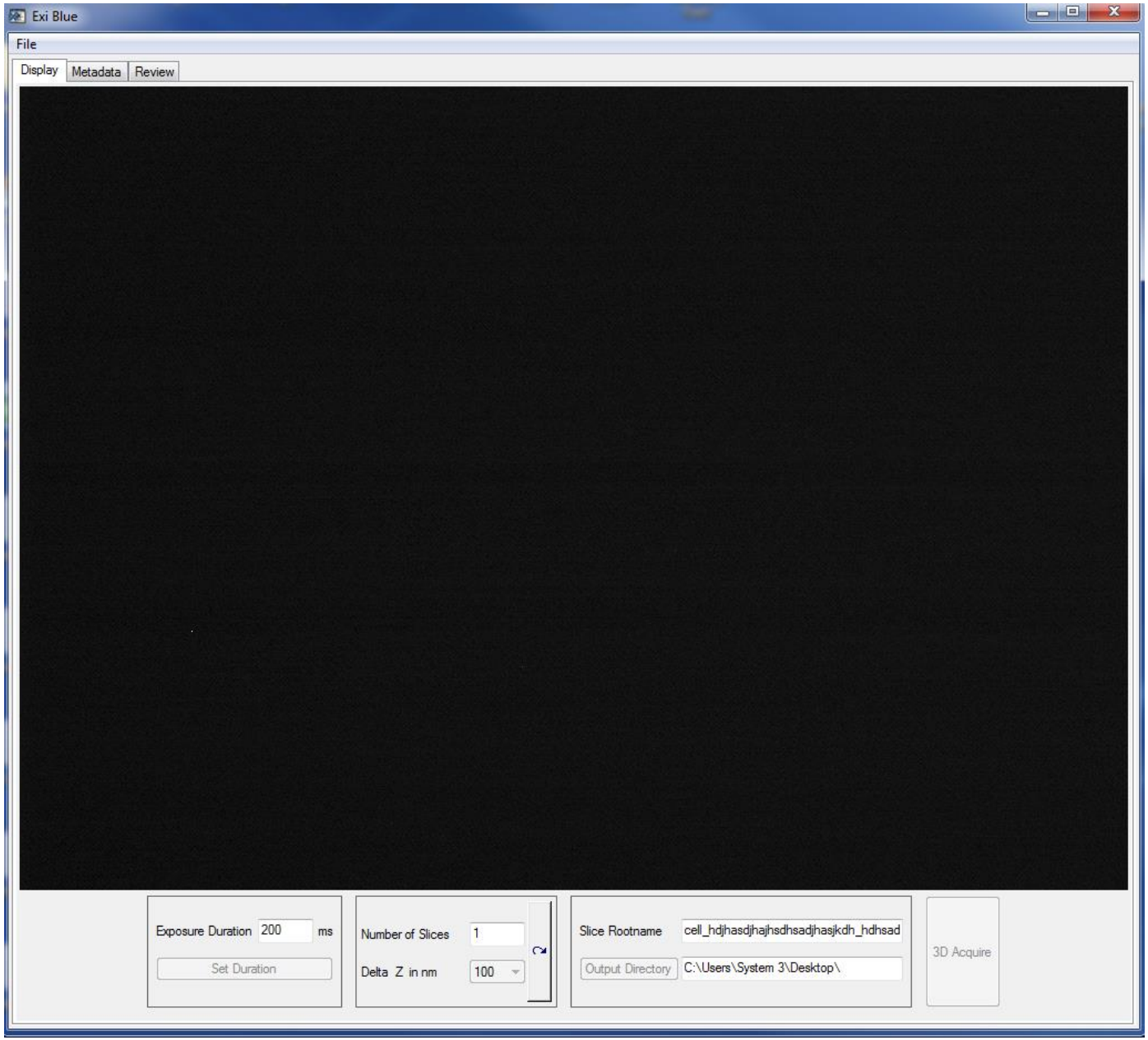
5. Click “Open” and the Acquisition window will appear.

*If you get a warning saying the camera is not found please check that the power is on for the Exi Blue camera or cycle power off and then on again.

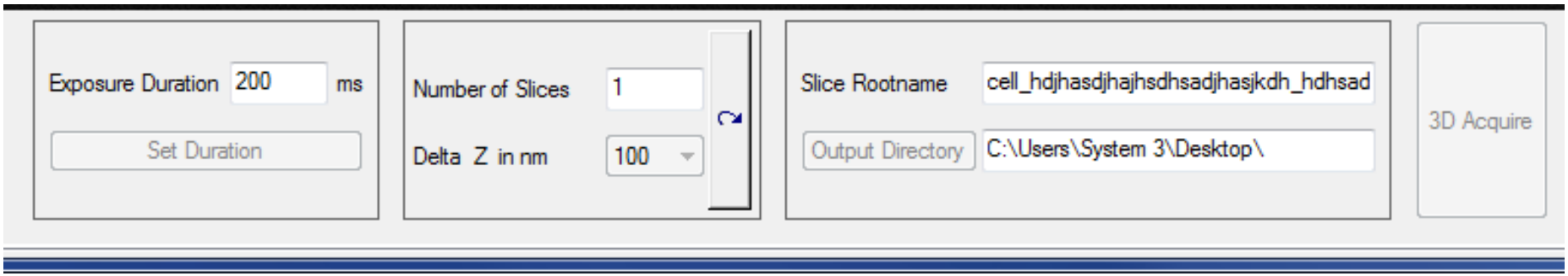
6. When the camera acquisition window opens initially the camera will need to cool to its set temperature of 0˚C.



7. When the camera has cooled the acquisition window becomes active and image stacks can start to be obtained.

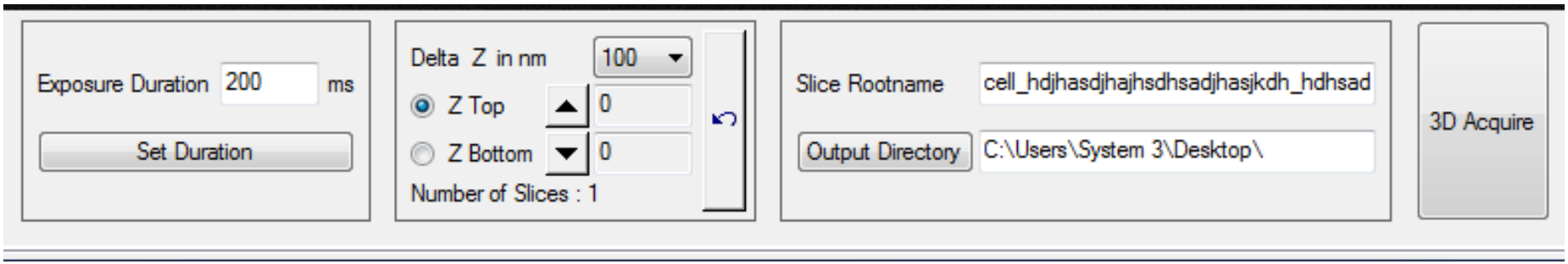


8. The camera acquisition parameters are as follows:



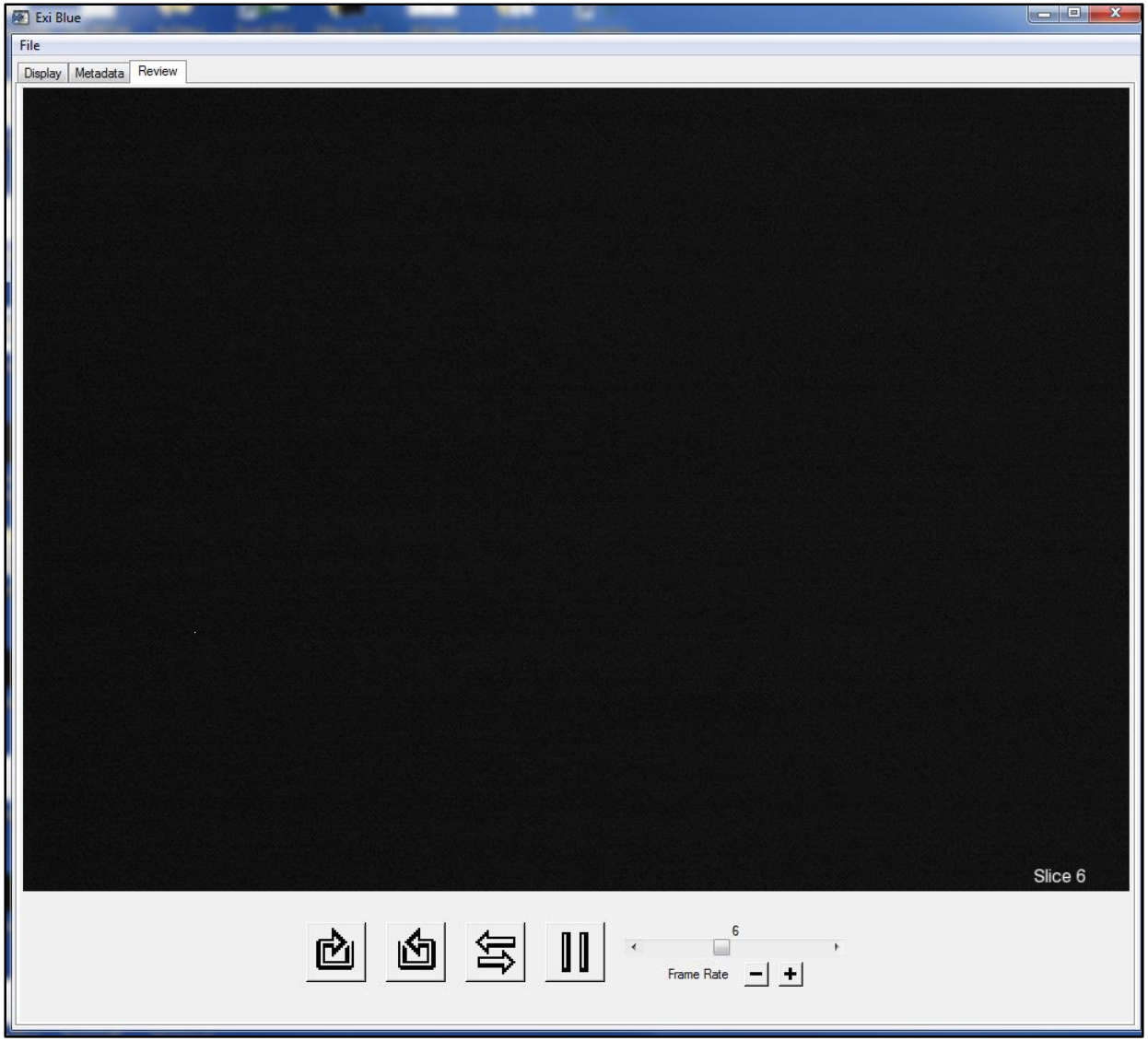
- "Exposure Duration" is exposure time in milliseconds.
- "Number of Slices" is the number of slices you want to set.
- "Delta Z in nm" is the slice spacing.
- "Slice Rootname" is the file name desired.
- "Output Directory" is the directory where you would like the stacks saved to.
- The "3D Acquire" button starts the collection of the stack.
- * All stacks are saved in the multi-.tiff format. This means a single file is saved as a .tiff but all images collected within the stack are embedded in the single file. Individual slices in the stack may be deleted later using the ImageJ software.

9. By clicking the arrow button next to the “Number of Slices” button you can change the way you set your top and bottom slices.

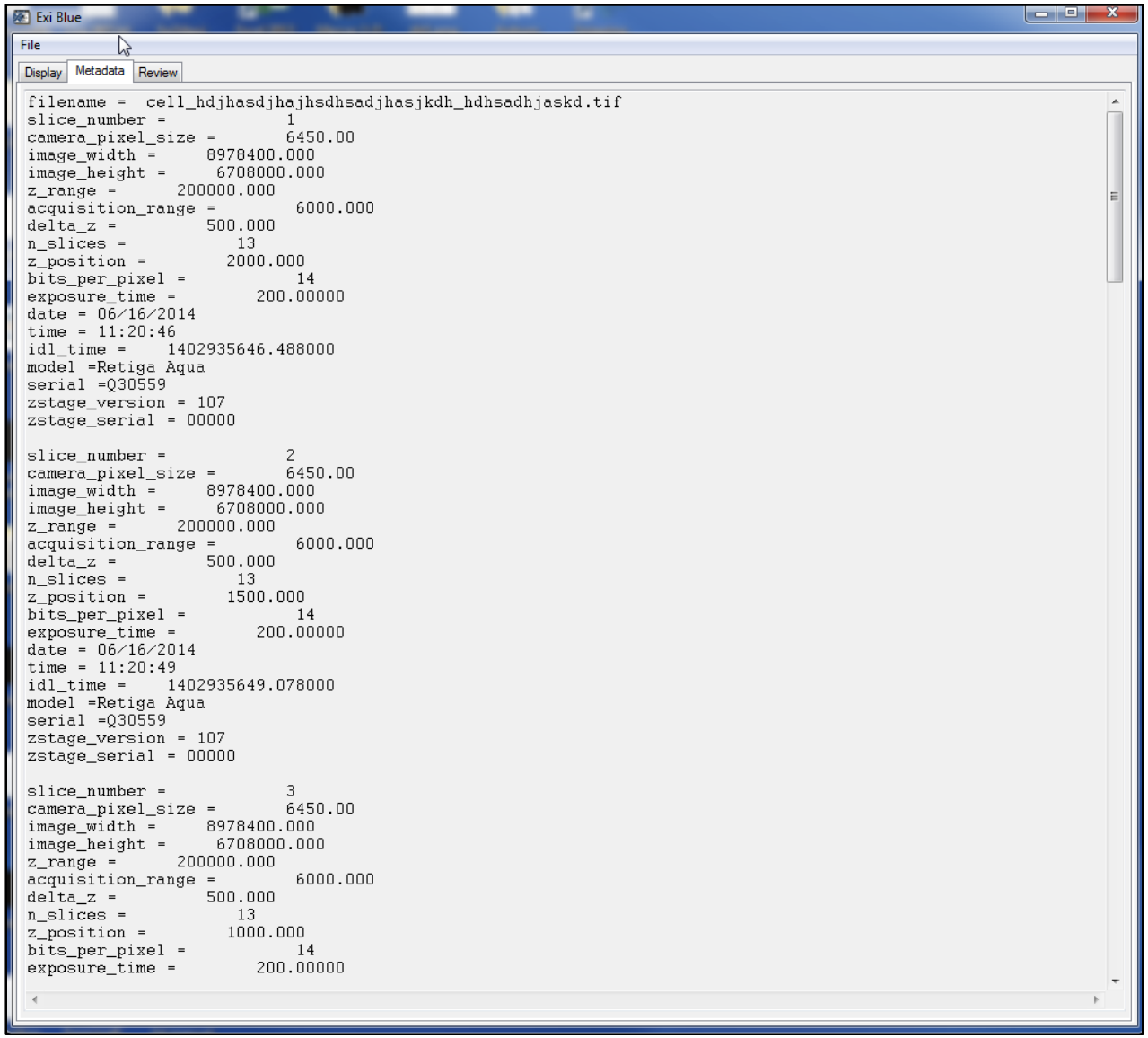


- You still use the “Delta Z in nm” option to set slice spacing. It is suggested that you should use the lowest number of slices possible for the best resolution in the Z direction. This would be 100nm.
- By clicking the up and down buttons next to the “Z Top” and “Z Bottom” radio buttons you can move the nano stage up and down in the spacing selected. For example if 100nm is selected in the “Delta Z in nm” parameter then each click of the up or down arrow moves the stage 100nm. This can be helpful in setting the Top and Bottom of the stack for your sample. This will automatically set the number of slices collected for the stack. We recommend that you manually focus the microscope to the center of the sample then use the arrow keys to set your top and bottom of the Z position. Click the “Z Top” radio button and click the up arrow to go to the top of the sample. Then click the “Z Bottom” radio button and click the down arrow to go to the bottom. The number of slices will automatically be set.

10. After a stack has been acquired you can click the “Review” tab at the top of the dialog box to review the stack. It will automatically start playing through the slices. You can click the pause button to stop sequence and click individually through the slices.

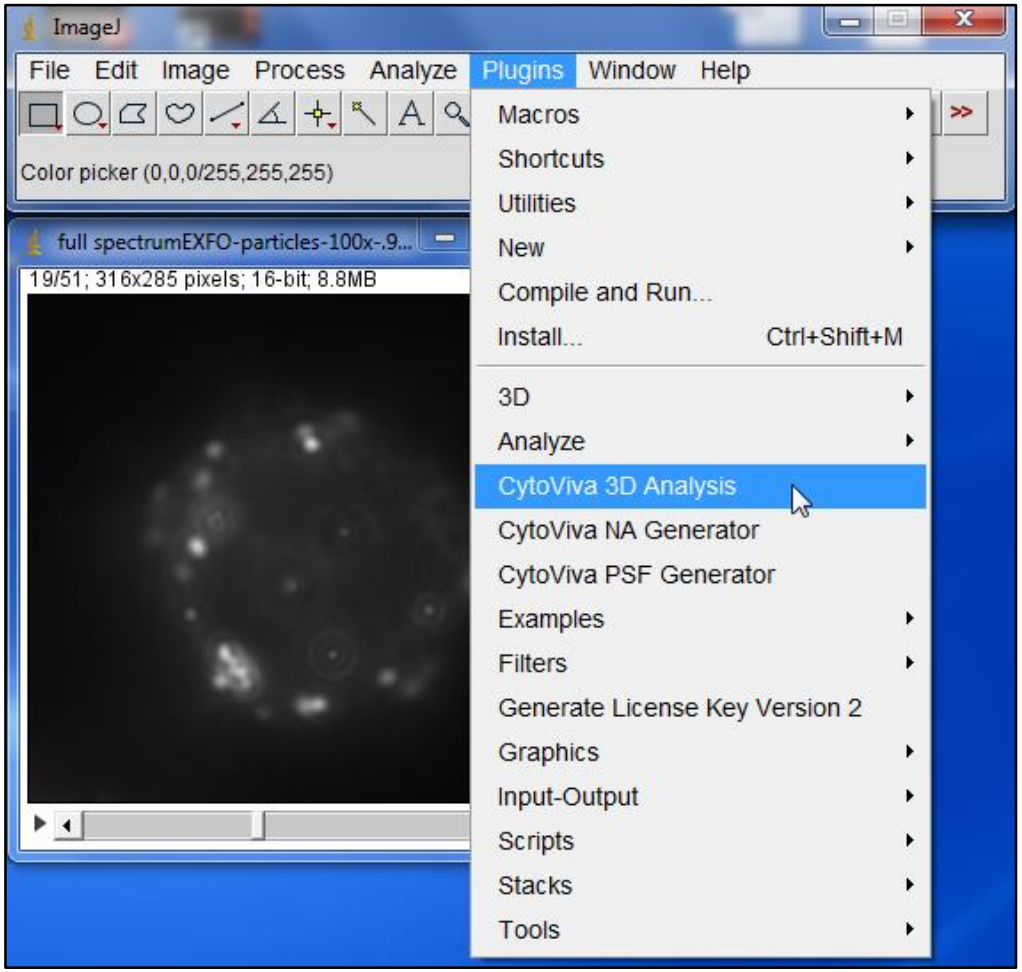


11. After a stack has been acquired you can review the metadata associated with the stack by clicking the “Metadata” tab. This shows all the information for each slice collected in the stack.



3D Image Analysis:

1. Once the stacks are completed you will open them using the ImageJ software. Once ImageJ is open you can use the “file, open image” dialog to view and process the stack. Stacks are saved as multi-stack .tiff files. This means one .tiff file has all slices collected in the stack. Then in “Plugins” menu select the “CytoViva 3D Analysis” plugin.



2. The “CytoViva 3D Analysis” plugin has 4 operations:

1. Just interpolate – This operation takes all slices and divides them up to make a single x,y pixel square in all directions. For example: at 100x a pixel in the X,Y spatial direction is 64nm x 64nm but could be 100nm in the Z direction. Interpolation will divide this single Z slice into roughly 2 slices to make each X,Y,Z pixel square. This operation automatically happens all of the operations but is available seperatley.

2. Just Locate Nanoparticles – This operation uses intensity differences in the whole stack to separate nanoparticles from other structures based off scattering intensities. It will count these objects and give a separate 3D stack of just the center of these particles.

3. Depth of Brightest Voxel – This operation performs the “Just Locate Nanoparticles plus takes all the other pixels in the image and makes them colored to create a “pseudo” surrounding (i.e. cell, tissue, etc.).

4. Deconvolution – This operation runs the deconvolution algorithm to the selected stack. For this operation a Point Spread function (PSF) will need to be generated. PSF generation is a separate operation and will be explained in the next section.

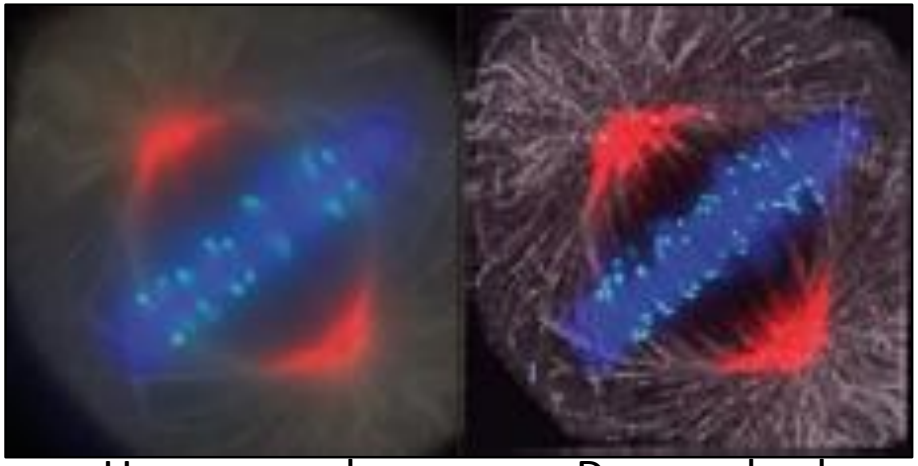
Deconvolution and Point Spread Function

Deconvolution: the term "deconvolution" is specifically used to refer to the process of reversing the optical distortion that takes place in an optical microscope to create clearer images. The usual method is to assume that the optical path through the instrument is optically perfect, convolved with a point spread function (PSF), that is, a mathematical function that describes the distortion in terms of the pathway a theoretical point source of light (or other waves) takes through the instrument. Deconvolution is performed digitally using one of several different algorithms.

Point Spread Function: ..or PSF describes the response of an imaging system to a point source or point object. The PSF of an optical device is the image of a single point object (rescaled to make its integral all over the space equal 1). The degree of spreading (blurring) in the image of this point object is a measure for the quality of an optical system. PSF's are generated by inputting the recording parameters of the optical system and sample parameters. Parameters like known wavelengths for fluorescence, Numerical Aperture of objectives, refractive index of media and oils, etc..

Deconvolution and Point Spread Function Cont.

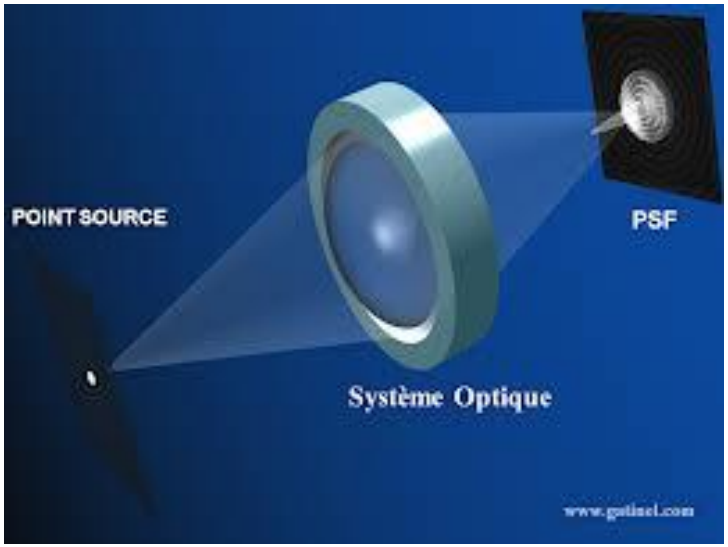
Deconvolution: The below image show the optical differences between an unprocessed image and a deconvolved image of cell division.



Unprocessed Deconvolved

Point Spread Function:

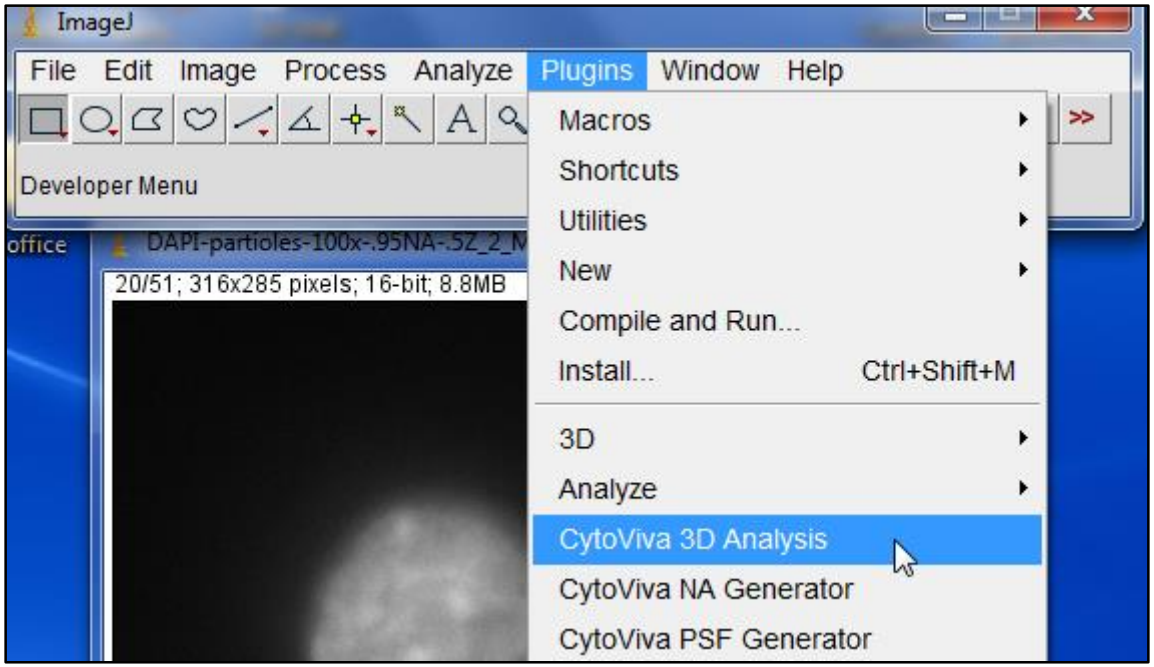
This diagram shows how a point source is imaged and the systems optics create an impulse response that is corrected by a theoretical PSF.



3. Generating a Point Spread Function or PSF:

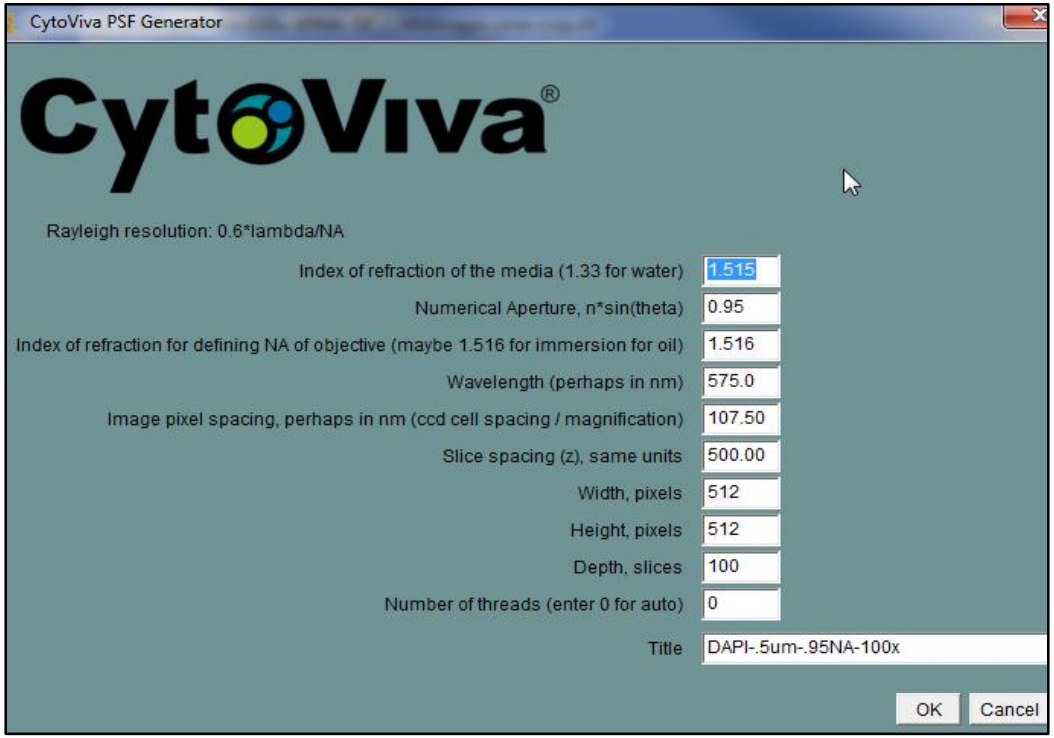
- A PSF needs to be generated to perform Deconvolution on 3D stacks and is unique to each sample based on the samples peak wavelength (fluorescent stain for example), the media involved (water, oil), the recording conditions such as objective magnification, Numerical Aperture (NA), camera pixel size, Z slice spacing, etc.

- To run the “CytoViva PSF Generator select it from the “Plugins” menu in the main ImageJ menu.



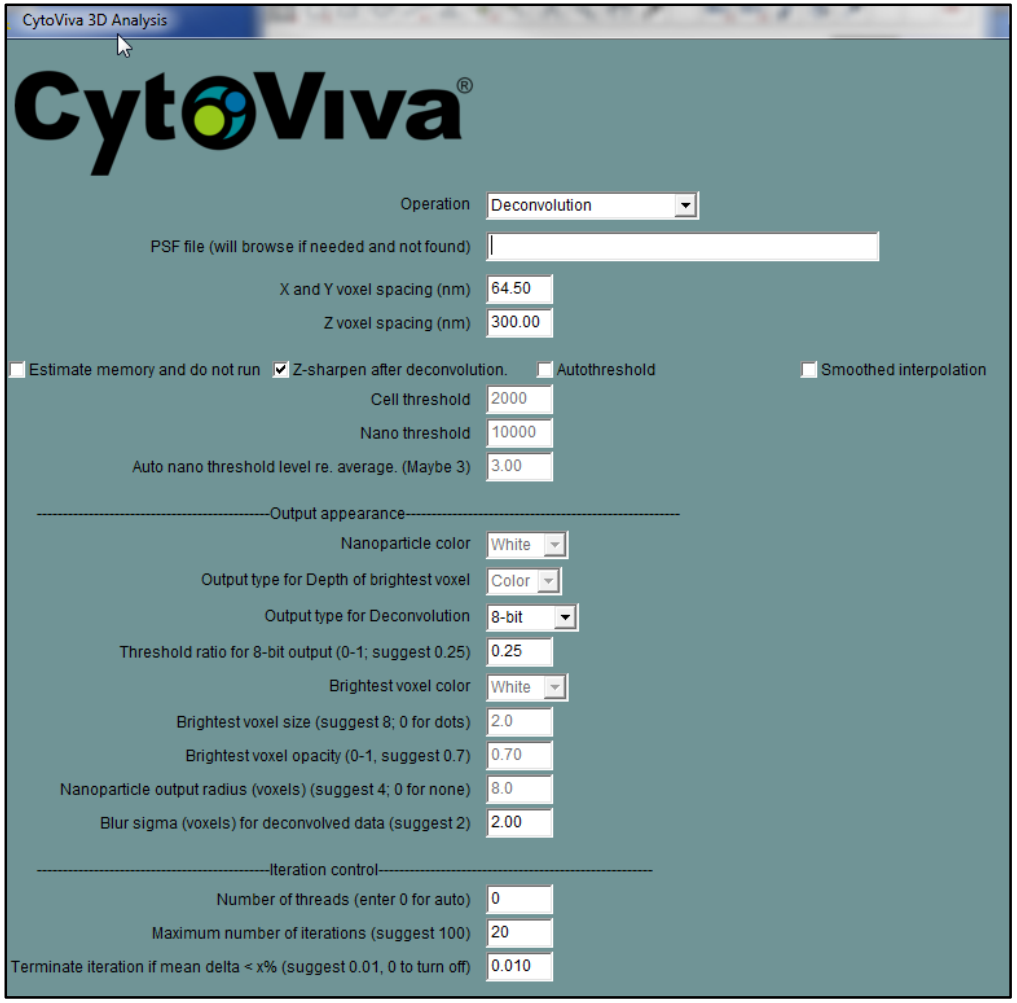
4. Using the CytoViva PSF Generator:

-**Index of refraction of media-** This is the refractive index of the media the sample is in. Usually will be water.
-Numerical Aperture- This is the NA of the optics used. For variable iris objectives you can set NA to lowest setting for certainty or use our "CytoViva NA Generator" plugin to calculate unknown NA.
-Index of refraction for defining NA of objective- This is for Oil immersion objectives such as 60x or 100x. For air objectives the NA would be 1.0.
-Wavelength- Peak wavelength in nm. For example a DAPI stain stack would be 460nm. For full spectrum stack it could be the peak of the lamp.
-**Image pixel spacing-** calculated by dividing magnification into CCD size which is 6450nm.
-**Slice Spacing-** the slice spacing of the stack in nm.
-**Width, Height in pixels-** The dimension of the PSF should be at least the size of the area. For example if you crop the image of one cell or section to 512x512 the PSF should be at least 512x512.
-**Depth in slices**- The PSF should be at least as many slices deep as the stack. So a 50 slice stack should have a PSF that's at least 50 slices.
-**Number of threads-** This should always stay 0.
-**Title-** This is what the PSF will be named. It's HIGHLY recommended that you name the PSF with all the information such as NA, wavelength, slice spacing, width, height, etc.



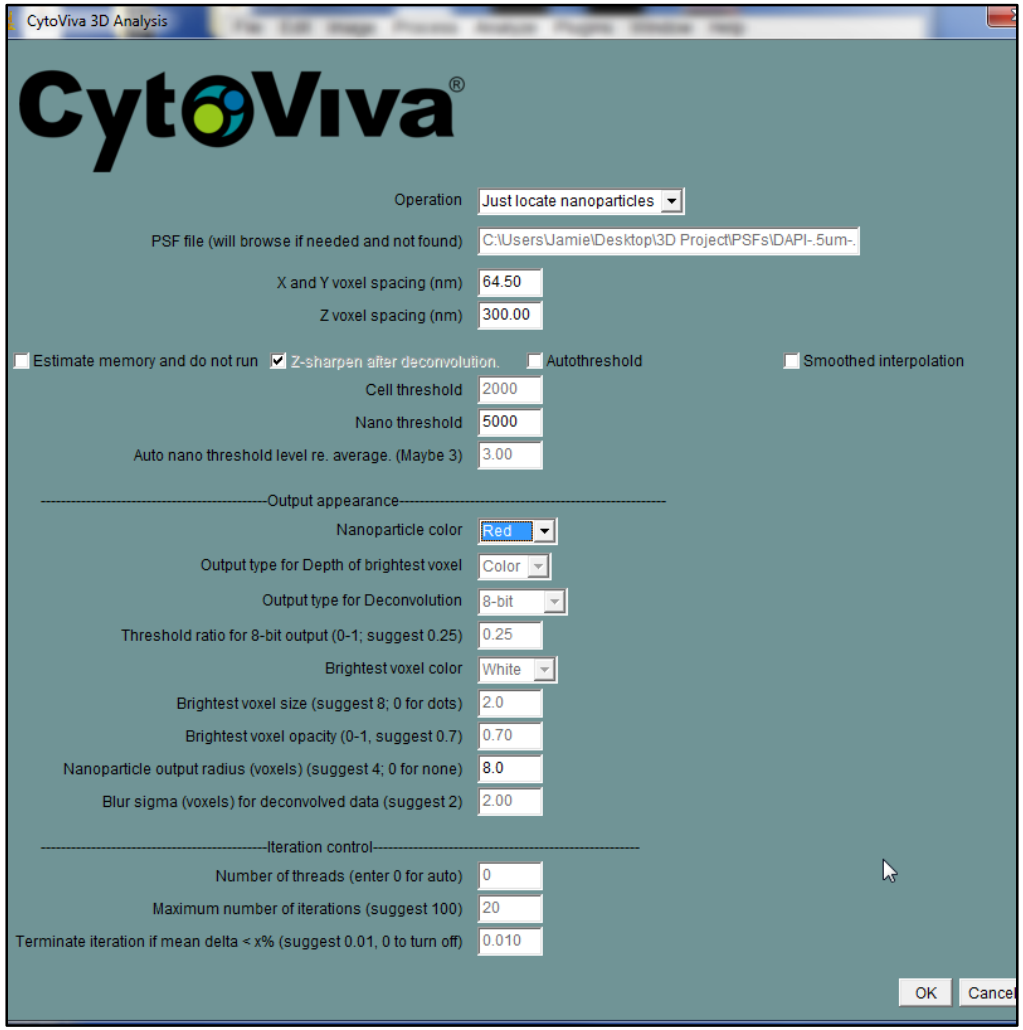
5. Using the Deconvolution Operation:*Options not relevant the given operation are greyed out!

-**PSF-** You will need to have a PSF ready to be used for the operation to run. Leave blank to browse for it.
-X and Y voxel spacing- This will be the size of a pixel in and X,Y direction. 64.5nm for 100x or 107.5nm for 60x for example. Again,camera pixel size/magnification.
-Z voxel spacing- the increment of spacing in Z set for the stack.
-Estimate Memory and do not run- This shows you the amount of memory needed to run the deconvolution routine without crashing.
-Z-sharpen after deconvolution- this applies a Z-stack sharpening routine to all stacks and is optional.
-**Smoothed Interpolation-** This smoothes the interpolated stacks and is optional.
-**Output for Deconvolution-** this sets the output for the deconvolved stack to 8bit or 32bit.
-**Blur Sigma for deconvolved data-** This applies a blurring routine to deconvolved data to smooth edges.
-**Number of threads-** This should always stay 0.
-**Maximum number of iterations-** The number of iterations is the number of times the algorithm is applied. The more iterations the more the data is deconvolved .
-**Terminate iteration if mean delta..-** This means no matter how many iterations is entered the routine will stop if the data processing does not improve more than .001%.



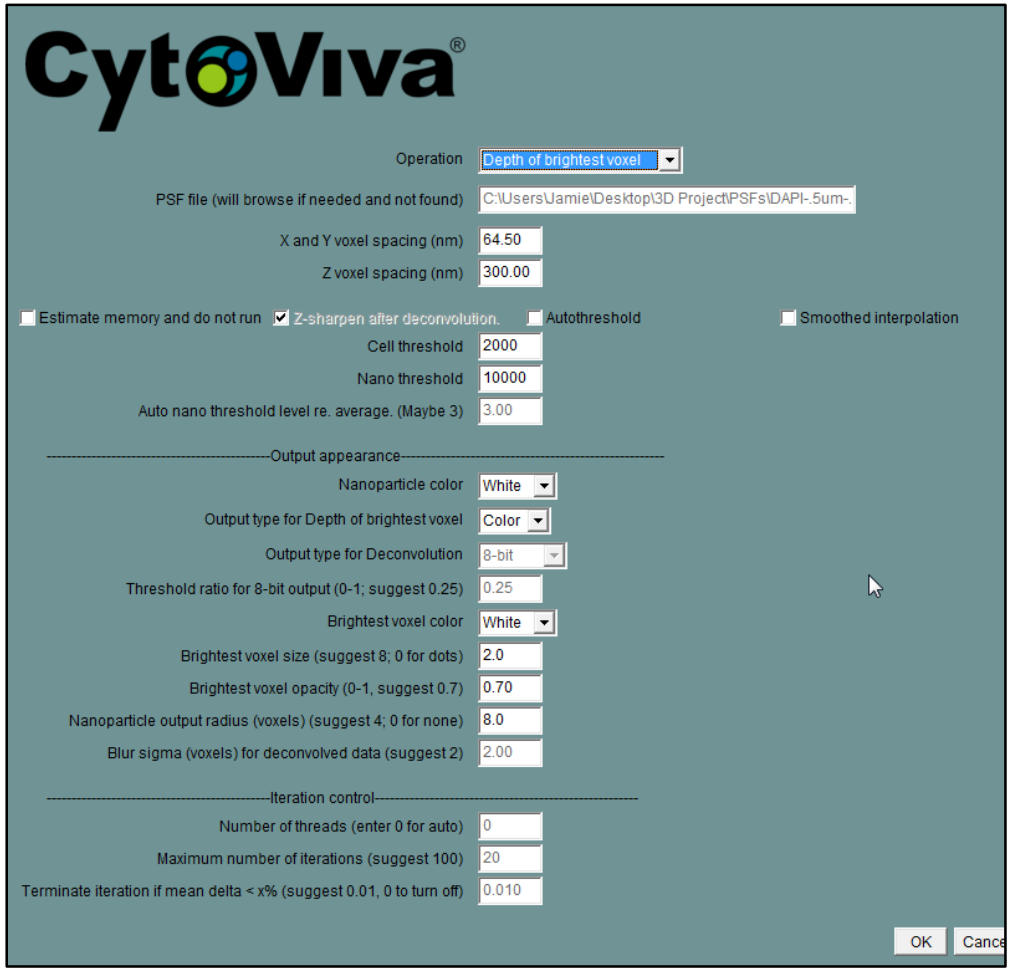
6. Using the Just Locate Nanos Operation:*Options not relevant the given operation are greyed out!

-X and Y voxel spacing- This will be the size of a pixel in and X,Y direction. 64.5nm for 100x or 107.5nm for 60x for example. Again,camera pixel size/magnification.
-Z voxel spacing- the increment spacing in Z set for the stack.
-Estimate Memory and do not run- This shows you the amount of memory needed to run the routine without crashing.
-Autothreshold- this will attempt to automatically set the threshold for the intensity of nanoparticles in the stack. It is recommended to uncheck this box and set the Nano threshold yourself.
-Smoothed Interpolation- This smoothes the interpolated stacks and is optional.
-Nano threshold- This the intensity point at which a pixel will be identified as a nanoparticle.
-Nanoparticle color- this sets the color of the pixels labeled to be nanoparticles.
-Nanoparticle output radius- This sets the number of voxels will represent a single nanoparticle.



7. Using the Depth of Brightest Voxel Operation:*Options not relevant the given operation are greyed out!

-X and Y voxel spacing- This will be the size of a pixel in and X,Y direction. 64.5nm for 100x or 107.5nm for 60x for example. Again,camera pixel size/magnification.
-Z voxel spacing- the increment spacing in Z set for the stack.
-Estimate Memory and do not run- This shows you the amount of memory needed to run the routine without crashing.
-Autothreshold- this will attempt to automatically set the threshold for the intensity of nanoparticles in the stack. It is recommended to uncheck this box and set the Nano threshold yourself.
-Smoothed Interpolation- This smoothes the interpolated stacks and is optional.
-Cell threshold- This the intensity point at which a pixel will be identified as structure other than a nanoparticle.
-Nano threshold- This the intensity point at which a pixel will be identified as a nanoparticle.
-Nanoparticle color- this sets the color of the pixels labeled to be nanoparticles.
-Nanoparticle output radius- This sets the number of voxels will represent a single nanoparticle.
-Brightest voxel color-this sets the color of the pixel labeled as cell or other structure.
-Brightest voxel size- This sets the number of voxels that will represent the other structure set in the “Cell threshold” dialog.
-Brightest voxel opacity-This changes the opacity if these pixels.
-Nanoparticle output radius-This sets the number of voxels will represent a single nanoparticle.

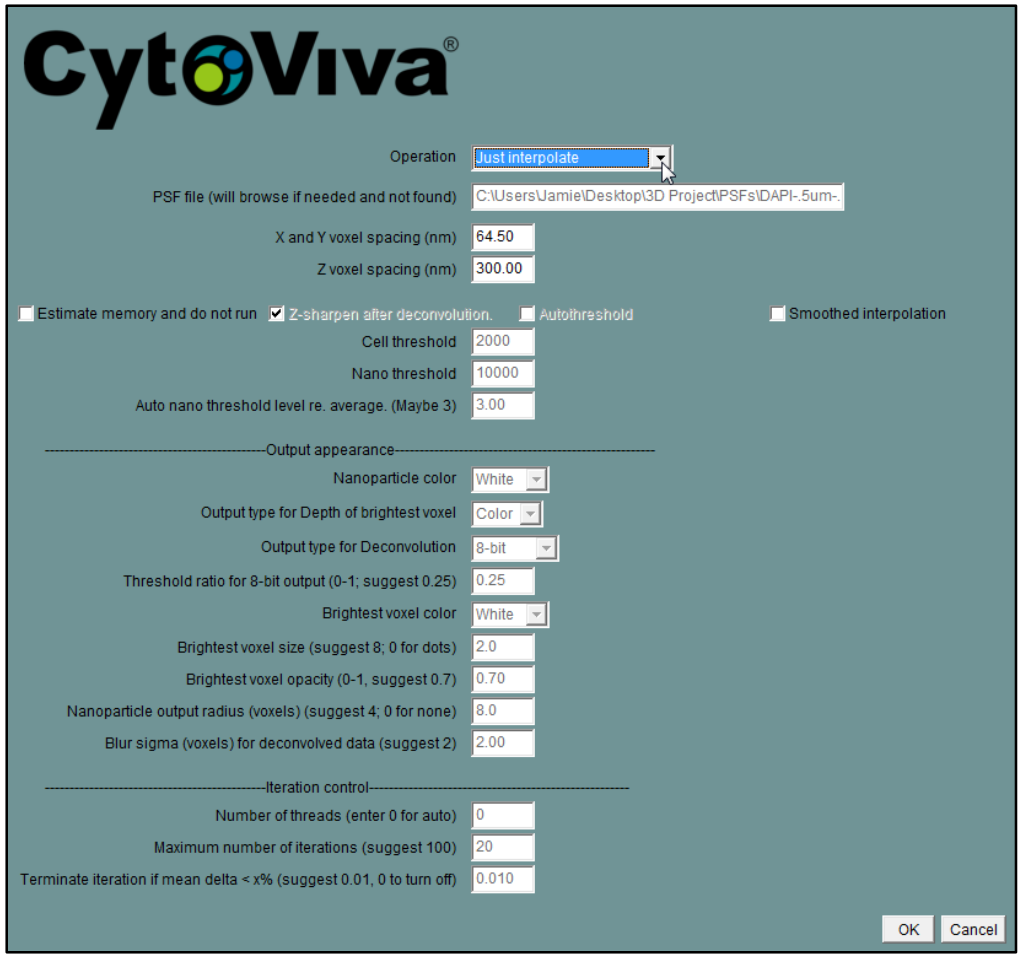


8. Using the Just Interpolate Operation:*Options not relevant the given operation are greyed out!

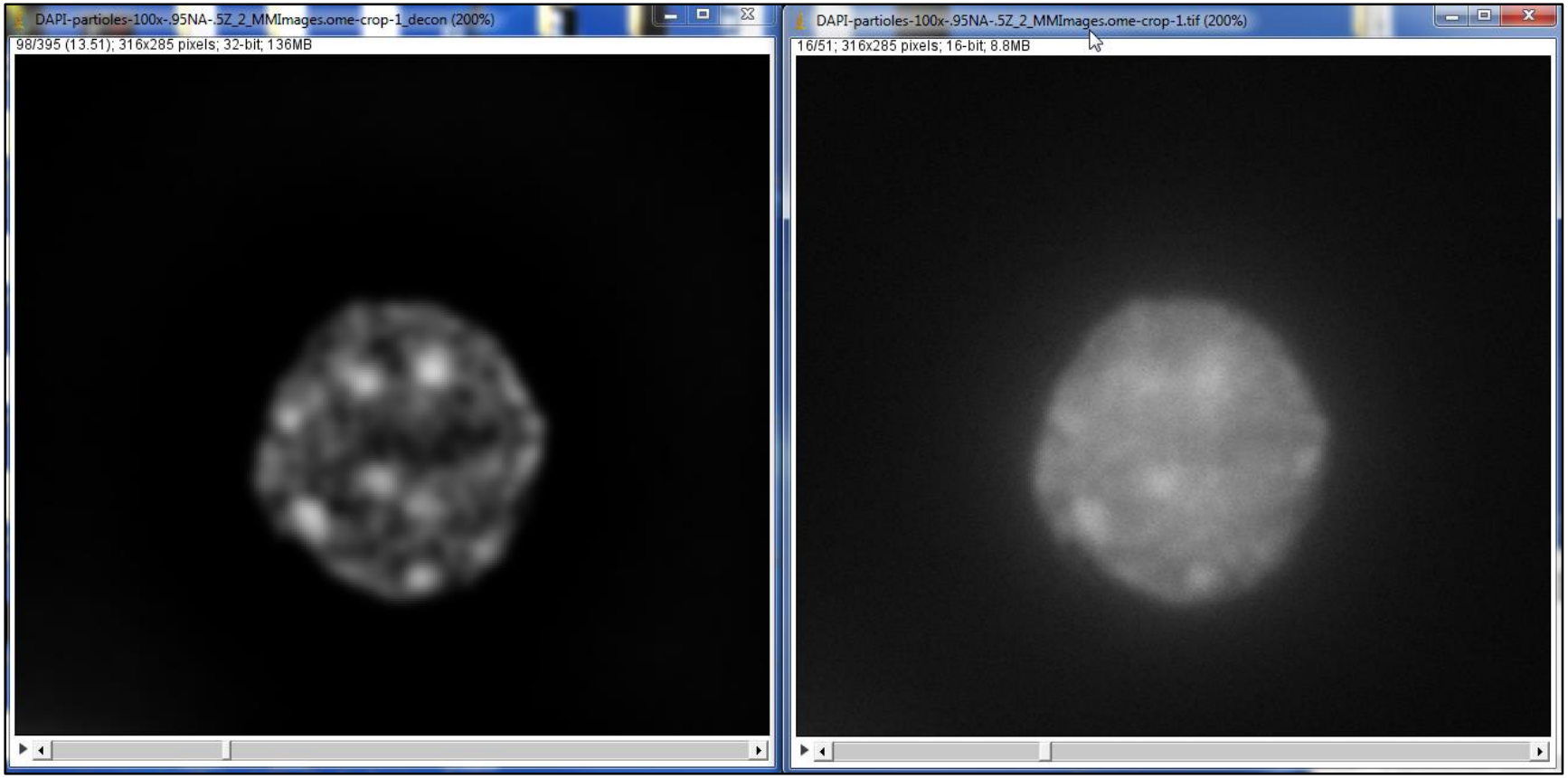
-X and Y voxel spacing- This will be the size of a pixel in and X,Y direction. 64.5nm for 100x or 107.5nm for 60x for example. Again,camera pixel size/magnification.

-Z voxel spacing- the increment spacing in Z set for the stack.

-Estimate Memory and do not run- This shows you the amount of memory needed to run the routine without crashing.



9. After the Deconvolution routine is complete it will open and you can see the difference the routine has made from the original stack. These stacks can also have other processing done such as sharpening, blurring, etc. that are found in ImageJ. You also may need to adjust the Brightness/Contrast. Just keep in mind that if you have a 32bit output you will need to change the file to 8bit for the brightness/contrast to take affect.



Deconvolved DAPI stain nucleus

Undeconvolved DAPI stain nucleus