APPENDIX: Image Analysis Instructions

1. Click on the StreamPix Icon on the computer desktop
2. Make sure power to the camera is ON and the light source to the camera is ON.
3. You should see the LIVE picture on the computer screen. If you see dancing coloured squares the data acquisition board is not selected. Under Hardware select Matrox→Meteor II MC

To take a Still Image (only to illustrate a blockage or some fact you would like to put in your report)

4. Under File select New Image Sequence→ tagged image file (tif)
5. Open a folder on the desktop that will contain your images from this lab and name your file.
6. Under Sequence select Snap one frame (or click the camera icon in the middle of the menu bar).
7. Check Folder to make sure image was recorded

To record particles as a live sequence

8. Under File select New Sequence in RAM (New icons will appear in the program)
9. To capture images 30 frames a second, go to File → Streampix settings…
    a. In the Recording tab, Stop recording "after 30 images have been captured"
10. Time stamp your images: select Plugins→ Norpix Time Overlay then select Relative and leave the default settings. Your images will have the time stamped on them relative to when you start recording. You will need this information to calculate velocities.
11. Press the Record button (orange hexagon) you will see the number of images being saved to RAM as total.
12. The recording should stop after 30 images. If it does not press the Record button again.
13. Make sure you note the speed of capture. It should be ~30 frames per second (fps)
14. You can view your files as a thumbnail under Sequence – View as Thumbnails. In this view you can see the time stamp for each image.
15. Save your files initially to the computer. Under Export save all your files (full sequence) as Tiff files to your Folder on the Desktop. Name your files with the channel you are measuring and place each channel data in a separate folder.
16. Click on the camera icon (far left) to return to the Live view

You can analyze your data in either of the 3 following ways, you need to download Image J (a free software). The simplest is #3 Image J using Manual Tracking Plugin.

1. Image J Instructions for Microvascular Chip Analysis – separate frame by frame

- Image J can be downloaded for free http://rsb.info.nih.gov/ij/download.html select windows, download first choice (6MB) put in Program Files or Google Image J and follow download instructions.
- Open image J then open the 2 files you would like to analyze
- To more clearly identify the individual beads in both files, mark them either by placing a circle around them – circle tool, right click → draw or with text tool call each bead 1,2, 3,… etc or a, b, c….
- Once the beads are clearly identified you can easily tell the distance moved when you merge the two images.
- To merge the two images →Process → Image calculator (Image 1 add Image2, create a new window and 32 bit).
- The resulting image should let you see how/where the beads have moved in the time between two frames.
- You now need to measure the distance moved. First you have to Set the Scale.
- With the line tool measure across the channel (this is a known distance)
- Analyze→set scale→ type in known distance→ type in um→check global
- Now the program is measuring in microns not pixels
- With your line tool measure the distance bead 1 traveled, then under Analyze→ Measure **or** Ctrl +M
- A Results table with lengths in microns should appear
- With the distance and time between frames you can calculate the velocity.
- Under file (in the Results/measurement window) you can save the results as an .xls file

2. Image J using an .avi file (movie) to track beads.

- Image J can be downloaded for free http://rsb.info.nih.gov/ij/download.html select windows, download first choice (6MB) put in Program Files or Google Image J and follow download instructions.
- You also need to download an AVI plugin from http://rsb.info.nih.gov.ij/plugins/
- Open Image J software
- Go to Plugins→input-Output-→ AVI reader (to make sure you have an AVI reader)
- Under File → Import → Image Sequence- the files of 1 series of data (e.g – one set of the 50um channel data) then click OK. It helps if the data is in separate folders.
- You now have all your images stacked together. Under File → Save As →Avi file, so you can go back to movie file when you need to.
- Open your .avi file, if you advance the scroll bar at the bottom you can see how your beads moved.
- **Set the Scale.** This converts pixels to microns. With the line tool measure across the channel (this is a known distance –use the width of the channel given in protocol)
- Analyze→set scale→ type in known distance→ type in um→check global
- Now the program is measuring in microns not pixels

- Select your + (crosshairs) tool and place it on the bead (if it is a doublet pick first or last –be consistent), then under Analyze→ Measure **or** Ctrl +M
- A Results table with coordinates x, y, z, and intensity will appear. Z is the frame #
- Advance to the next frame and move the crosshairs to the new center of the bead and Measure (Ctrl+M).
- Repeat the process as you track each bead for at least 10 beads. The more beads you analyze the more accurate your velocities will be.
- Under file (in the Results/measurement window) you can save the results as an .xls file
- With the distance and time between frames you can calculate the velocity.
- Distance is the square root of $(\Delta x^2 + \Delta y^2)$
- If you use 1 excel file for all the beads in your movie be careful to note when you switch to different beads.

3. Image J using Manual Tracking plugin

- Image J can be downloaded for free http://rsb.info.nih.gov/ij/download.htmlselect windows, download first choice (6MB) put in Program Files or Google Image J and follow download instructions.
- You also need to download an Manual Tracking plugin from http://rsbweb.nih.gov/ij/plugins/track/track.html
- Open Image J software
- Under File → Import → Image Sequence- the files of 1 series of data (e.g – one set of the 50um channel data) then click OK. It helps if all the data is in a separate folder.
- You can covert to an avi file if you have .avi plugin (see Method 2)
- **Set the Scale.** This converts pixels to microns. With the line tool measure across the channel (this is a known distance –use the width of the channel given in protocol)
- Analyze→set scale→ type in known distance→ type in um→check global
- Note the conversion factor of pixels to microns
- Click on the image sequence you just opened
- Go to Plugins→ Manual tracking
- In the *Parameters* panel, set the time interval (the time interval between each picture frame), the xy scale (put in the conversion factor of pixel to microns –this is 1/(value in set scale), and ignore the z scale
- To start a new track, click on "Add track". Then click on the bead you are tracking in each frame as the program goes through the image sequence.
- After tracking one bead, click on "Add track" again to start tracking another bead.
- A track can be removed by selecting the appropriate track number in the central listing and clicking the "Delete track n°" button.
- You want to keep a note of the order you track the beads in since Manual tracking puts all tracks (labeled with track number) in one excel file. Also note that the parameters are now hidden. To show parameters, tick the "Show parameters" option.
- The resulting excel file contains the coordinates of each track and its velocity.