This SOP covers all of the procedures needed to determine *displacement* fields from tissue volume images obtained using two-photon microscopy in Dr. Alex Huang's lab (6402 Wolstein Building). The "Materials Required", "SAMPLE MOUNTING", and "IMAGING" sections are substantially the same whether the end point is point tracking or image correlation. The only difference is that for image correlation the cells do not need to be stained with ethidium bromide.

SOP: Imaging, Image Processing and Analysis – Displacement Fields

Prepared by: Joe Heebner   Bryan Benson
Contact Info: 609-571-2386
                 Jeh165@case.edu   blb75@case.edu

Materials Required:
Test apparatus
2 Newport Linear Actuators and their respective Conex controllers
Conex AC/DC Power adapter and supply cable
Conex DC jump cable (~5" in length)
2 USB to USB-mini A cords
Power supply for load cell
Windows computer with CONEX-CC GUI installed
Windows computer with LABVIEW acquisition program installed for load cell data
Cartilage Sample (preferably several)
Metric hex key set
Thin-style box wrench (15/16 mm)
Razor blade/ scalpel
Crazy Glue or Tissue Glue
Fluorescent Beads
Windows computer with Imaris installed
Windows Computer with MATLAB installed (can be the same as the Imaris or other computers)
Ethidium Bromide nuclear stain, diluted 1:2000
Latex gloves
Phosphate-Buffered Saline

SAMPLE MOUNTING

I.   Setup
       a. Ensure that all actuators are securely mounted within the apparatus.
       b. Back off the compressive-axis actuator by hand so that there is enough room to manipulate the sample and holder without collisions.
       c. Plug the CONEX power supply in, insert the other end to the DC IN port on one of the controllers. Use the jumper to connect between the DC OUT port on the controller connected to the wall supply and the DC IN port on the other controller.
       d. Ensure that actuators are not going to crush sample.
              i. After supplying both actuators with power, and plugging both of them into the controlling computer, initiate the CONEX-CC GUI application.

      ii.   Use the discover button and make sure both actuators are shown, then proceed into the application's main screen. Both controllers should be in a NOT REFERENCED state with an orange display on the controller LED.

      iii.   In the diagnostics tab on top send the following set of commands to both actuators in this order. Note which controller (compressive or shear) shows up as X, and which as Y in order to avoid erroneous boundary displacements later.

1. 1PW1 (This sets the actuator controller in configuration mode)
2. 1HT1 (This sets the target location for initialization at the current position of the actuator)
3. 1PW0 (This exits configuration mode)

      iv.   Initialize both X and Y actuators.

   e.   Plug in and connect the power supply box to the load cell. Connect load cell to LABVIEW computer and open program "aftdqxmvi" (or similar name) from the top of the recent programs list. If program fails to open, try other programs of the same name.

II.   Sample Mounting

   a.   Using a hex key, unscrew the mounting bolt from the sample holder and remove the sample holder from the apparatus.

   b.   Ensure that sample is of a size that will not made contact with the bottom of the titanium 'bathtub' within the apparatus after mounting.

   c.   Coat the end of the holder with cyanoacrylate glue and dust with fluorescent beads, then attach sample to holder, ensuring that sample has material above the top surface of the holder.

   d.   Apply cyanoacrylate glue to the load-cell side sample mount.

   e.   Replace stationary sample holder and secure firmly with hex bolt. Bring sample into complete contact with load cell-side mount.

      i.   Use the "Move Relative" option in the Conex-CC GUI to apply displacements. Units are millimeters, so unless the gap is large, ensure that numbers are in the tenths (100s of microns) or hundredths (10s of microns) to avoid overloading the sample and load cell.

      ii.   Monitor the load cell to ensure that it is not overloaded (Numbers over 50 are of high concern)

   f.   Once the glue has cured with the sample attached to both mounts, then and ONLY then use a flat, sharp blade to slice the excess cartilage off at the plane of the top surface of the mount. It is important to wait until glue has cured, or the glue layer will inhibit proper imaging under the microscope. Hold the blade at an angle between the axis of compression and the axis of shear to make an optimal cut.

III.   Insert the apparatus into the two-photon microscope.

   a.   Ensure that all actuators remain connected to the controlling computer. If they have become detached, reconnect them and repeat the steps in Imaging section I., iii. Numbers 1-3 , and I. iv. to ensure that they don't move again during imaging. Furthermore, ensure that the computer controlling the actuators will not die, shut down, or fall asleep if left idle for extended periods.

   b.   Inspect the view of the sample. Ensure that the stationary holder (henceforward referred to as the zero-displacement boundary) is within the field of view. If sample appears thoroughly unsatisfactory, mount a new sample according to the instructions above.

   c.   In the LABVIEW interface, zero the force and moment readout by toggling the "bias" button prior to applying any displacement to the sample.

   d.   The sample is ready for displacements to be applied and imaging to be done. **DO NOT APPLY DISPLACEMENTS OR OTHERWISE MOVE THE SAMPLE DURING IMAGING AS THIS RISKS DAMAGE TO THE MICROSCOPE AND WILL INVALIDATE THE IMAGE STACK**.

IMAGING

I.   Stepping and Imaging
   a.   Decide on image stack parameters, size as fields of view and resolution. After parameters have been set, fix them to remain constant during imaging. It is usually wise to start a (very) few microns under the actual surface of the sample to ensure that the field of view does not have wasted space. Gain compensation to ensure good imaging of nuclei deeper inside the sample may be added here to save trouble in processing.
   b.   Take an image stack prior to applying any displacements.
   c.   Note the force and moments in each axis from the LABVIEW display.
   d.   Apply each displacement using the "Move Relative" option in the Conex-CC GUI. Use small displacements on the order of 1% of the sample thickness (usually ~8-10 microns). Larger displacements may cause inaccuracies in the analysis and smaller displacements will lead to bleaching of the stain before significant total displacement has occurred.
        i.   Monitor the force on the load cell and when the change per minute is less than 15% of the jump in major force or major moment, it is reasonable to commence imaging of the new stack.
II. Separation
   a.   Using LAS AF Lite (Available for free online) separate the image stacks into separate .lif files before moving to processing.

IMAGE PROCESSING

I.   Convert .lif image stacks into imaris files for tracking work:
   a.   Use imaris file converter (third row down on desktop) (drag and drop .lif files) (can be done all at once)
   b.   Press "Start" at the bottom right of the window
II. Concatenate the image stacks as time slices for later displacement analysis
   a.   Open the first Imaris file(likely zero compression or zero shear stack)
   b.   Use the "Save as" and select a new name to avoid overwriting the single stack file.
        Edit… Add time points (the other imaris files). Add these files one by one to avoid out of order errors
III. Prepare to do spot analysis
   a.   Select spots (orange group of circles icon, top left corner)
        In the spots menu (bottom left corner), select the following options:
   b.   Do whole frame (don't 'Segment only a Region of Interest'), Do not select different spot size, yes track over time.
   c.   Under properties (top left), use crosshairs to get measurement points. Shift click to place measurement points, use measurement as estimate of cell size (not terribly important).
   d.   Use estimate to proceed to next step of tracking; don't worry about filters until step 6/6 except to make sure that all spots are in all frames (set filter to far right based on track duration). Imaris assigns arbitrary time gaps to displacement steps and selecting only those with the maximum time value ensures that all tracks are across five frames.
   e.   Autoregressive motion
   f.   Measure a rough max distance per frame, gap size zero
   g.   Edit > Preferences > Display > Unselect show relative time

IV.   Set up filters:
   a. Ensure again that all spots to be tracked are in all frames.
   b. Use location filters to eliminate cells who pass out of the volume scanned under the microscope (remove the extreme z values)
   c. Use the track straightness parameter to eliminate cell traces that are clearly algorithm misfires (if there are traces that look like a lightning bolt, this is one of the filters that should be applied.
   d. Use the 'quality filter to ensure that only spots that are actually chondrocytes are tracked.

V.   Prepare images and export data
   a. Manually adjust screen size in snapshot mode to get image the way you want it; otherwise it will not be as expected.
   b. Check to make sure it shows up as planned.
   c. Export all data. Click graph icon under relevant spots tracks and click the floppy icon in bottom right of panel.

VI.   Curate Data:
   a. Elimination of drift is done by finding traces of zero-displacement spots (can use fluorescent beads in the superglue during the mounting process) and subtracting either average of all or a select few of those track displacements (which, relative to the stationary apparatus are zero, but due to shifting of the apparatus and microscope drift will not be) from the displacements computed for the cells: this is written into the MATLAB analysis code, but the means for finding those traces is the same as the spot tracing done for the cells.

DATA ANALYSIS

I.   Import the Position .csv file (not trackPosition) from the Imaris data to the command window in MATLAB.
   a. Ensure spreadsheet looks correct; change import range to exclude the last (bottom) row that has numbers in it. Due to a quirk in MATLAB's indexing system, this is necessary to include all of the data without getting NaN (Not-a-Number) errors.
   b. Check any variable just imported in MATLAB to ensure all data was successfully imported. If not, change import range as described in Matlab code. Ensure that last ID tag matches data.

II.   Now import position for reference spot (same procedure applies to the range listed for import)
   a. This should give position X1 Y1 and Z1 with Time1 and ID1 data. In the event that multiple spots are used:
       i.   Average all of the reference positions for each axis and stack (frame)
       ii.   Save the averaged positions and the Time1 and ID1 data for the first reference spot
       iii. Clear X1, Y1, Z1, Time1, and ID1 data
       iv.   Rename the averaged position data and place the single-spot Time1 and ID1 data where they originally were.

III.   Run the MATLAB file. A surf plot should be output. If there are any errors, consult the code comments to ensure all inputs are acceptable.

IV.   It is possible to save detrend_position_xyz as .mat file for later analysis or concatenate the data to make it easier for the statistics folks. The concatenation is written into the MATLAB code.

TROUBLESHOOTING

I.   Setup, Mounting, and Imaging

      a. If the controllers exhibit unexpected behavior, consult the documentation for Conex-CC controllers (may be found at http://search.newport.com/?x2=sku&q2=CONEX-LTA-HL under the "Literature and Downloads" tab).

II. Processing and Analysis

      a. If errors occur before running the MATLAB analysis code, consult the documentation for the program at hand.

      b. If errors occur during the MATLAB code, ensure that inputs are from the specified files. If errors persist, use the contact information supplied at the beginning of this document to clarify further.