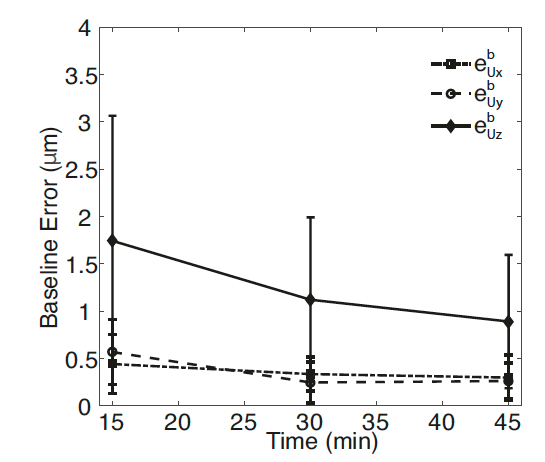
**Protocol for DVC displacement and strain error study:**Dan Midgett 09/17/2018

Description: When choosing imaging, image processing, displacement post-processing, and strain calculation parameters, it is important to have a standard to compare to optimize these settings. For this purpose, we use the displacement and strain error summary results stored in the variable *aSummaryALL* for a correlation error analysis of an image set with average quality, exported by the Matlab functions:  
*process\_results\_correlationErrorStats3Strain.m   
process\_results\_BaselineError.m*In this example we will be referring to the post-mortem mouse ONH imaging studies and Clinical OCT imaging of human ONH as examples.   
This variable contains a vector of error statistics as shown in the header labels in the excel file: “Mouse LC Error Summary”. Four error measures are summarized: Bias or average error, uncertainty or standard deviation of error, absolute mean error, and the standard deviation of absolute mean error. These error statistics are summarized for displacement and strain in the directions X, Y, and Z, or for UX, UY, UZ, and EXX, EYY, EXY, and EZZ. For the baseline imaging errors (a study which measures only creep errors between imaging sets) error data is only present for UX, UY, UZ. For this analysis we will mainly focus on analyzing how the bias or absolute mean error changes as we change parameters. The goal is to find settings that improve the average displacement or strain errors. Please keep in mind that this is not an exact process as you can’t realistically test everything, and thus can require some iteration to do efficiently for each new study. Also, keep in mind that for our correlation error analyses, we have applied a constant strain. For this reason, certain settings (such as the Gaussian smoothing window size or strain fitting domain width) will always have lower error the larger we make this parameter. For these or similar settings, we want to find the smallest settings for which large improvements are still being made (or the inflection point in the error curve). You could also choose to cap the upper size of these parameters if there is some good reason to do so (such as a strain resolution requirement). Ideally you want these kinds of settings to be as small as possible, yet still smooth out the influence of error spots or regions of poor correlation. Below we present a protocol for optimizing several different kinds of parameters. For imaging settings, we want to investigate the error due to imaging, or baseline displacement error and minimize it. To improve image processing settings, displacement error due to a numerically applied displacement and strain is the important metric to monitor, as you want to improve image contrast and subsequent DVC correlation accuracy. For displacement processing parameters and strain calculations, strain error is the most important metric to monitor and minimize.

**Protocol: Optimizing LSM Imaging Settings:**Description: When imaging with an LSM, several quality options are present to optimize. For instance, if you are imaging in two-photon imaging mode, the excitation wavelength can be varied. For our experiments with SHG and TPF, a 2P excitation between 780-800 nm is optimal for exciting collagen. We found that in our specimens an excitation of 790 nm worked well. In addition, you can change the imaging gain on your image channels to make them brighter or darker or change the digital slope or offset to shift the histogram of the captured image. In addition, you can change imaging settings to shift the image acquisition speed and turn on line averaging or bidirectional imaging. Since tissue creep during imaging is the major limiting factor in our resolution, we have in the past chosen settings that minimize the image acquisition time and error due to creep. This includes holding the specimen for a while at the desired IOP until creep becomes small and linear. We also image with the fastest imaging speed, use bidirectional scanning, and do not average. The effect of this choice is smaller creep magnitude, but dimmer images with more noise due to the fast scan speed. The image brightness will vary from image to image, so the optimal gain must be estimated for each specimen individually. I recommend turning on the option that shows clipped pixels in the image (255 or 0) and sliding the gain until you have no zero intensity pixels in the image (a background of slightly above zero). You can then increase the gain until only a few pixels of your brightest features have 255 intensity pixels. It is important that only a few small pixels have maximum brightness or you will lose contrast that could be used for correlation.

These imaging settings should not need changing in the future; however, the general protocol we followed to obtain them is the following:

1. Capture an image stack containing your specimen using the desired imaging settings you want to test
2. After the image stack is complete, immediately acquire another one (we will call this our duplicate image stack)
3. Vary the imaging setting you wish to optimize in increments (example: varying equilibration time) and acquire duplicate image sets (Steps 1-2) for each setting
4. Enhance the image as required, and save as a .mat file (see DVC analysis protocol)
5. Correlate the first image set with the duplicate image set taken at the same IOP for each individual imaging setting variation
6. Once the DVC run is complete, process the displacements for each imaging setting using the DVC analysis protocol, and run *process\_results\_BaselineError.m* to summarize the displacement errors
7. Refer to the summary variable aSummaryALL to see the bias and absolute average error due to creep or correlation error. Copy this vector into an excel file so you can compare the results side by side as you vary settings.
8. Generate a curve showing the variation of the bias and absolute average error with the imaging settings to justify your choice of settings.   
     
   Notes: When it comes to imaging settings, image quality and imaging speed matter most. Essentially we want to image at the fastest imaging speed we can manage without losing DVC correlation due to poor quality. In our experiments we found the best resolution (lowest displacement errors) to correspond with the fastest imaging speeds and made up for the loss of imaging quality with custom contrast enhancement and image processing.   
     
     
     
   *Figure 1*, baseline error in X, Y, and Z, evaluated at 5 mmHg for different equilibration times, showing that the error is subpixel and nearly constant after 30 minutes in the post-mortem human ONH. The error bars indicate 1 standard deviation.

**Protocol: Optimizing Image Processing Settings and Correlation**Description: Once the image is obtained, we need to determine the best way to enhance the contrast within the image for DVC correlation and the best settings for DVC correlation. In general, background noise and background signal is always a bad thing, and if it can be removed by processing this is ideal. This is because the presence of noise can obscure the finer details in a structure you are imaging. However, even if the noise is in the background, this can also affect your ability to segment the features from the background if the background signal is too bright. It can also making picking an appropriate correlation coefficient threshold (used to segment areas with clear features from areas corresponding to image background) more difficult. In addition, we are want to use contrast enhancement to equalize the brightness of features across the volumes, ensuring that features look similar in subsequent images and can be easily tracked. Brightness changes can occur as features tilt, rotate, or if debris or air bubbles pass between the detector, and these should be removed with contrast enhancement for best correlation. In the case of debris or air bubbles, just be very careful to keep the water column and specimen clean and this shouldn’t be an issue. If you see a shadow appear during imaging, air bubbles or debris are often the source of them. Contrast enhancement should only be conducted after noise is removed or the noise will also be enhanced so the order of optimal contrast is as follows: noise removal techniques, then contrast enhancement techniques, then gamma shifts or other histogram manipulations to tone down background intensity.   
  
The techniques you use will mainly be similar or identical between LSM images, however, if you use a new kind of image (such as OCT) the settings will need to be reoptimized for these kinds of images. New imaging modes or speeds may also need to have the signal to noise ratio for Huygens theoretical deconvolution reoptimized.

The protocol for optimizing image processing settings requires a numerically applied displacement error study to test the contrast quality, and is as follows:

1. Start with two duplicate imaged volumes (see previous protocol)
2. Enhance both image stacks using your desired image processing protocol, and vary the setting you wish to optimize (either image processing setting or DVC correlation setting) in increments.
3. Run the virtual\_displacement.m matlab file, and enter your first and second volumes as the reference and warped volumes to process in the file. This will apply a numerical displacement, simulating an RBM and constant strain, to the volumes and rename them. *Because we used two duplicates, realistic imaging issues such as changes in noise and background content between scans and creep are taken into account and this allows for a more realistic test of the efficacy of the contrast enhancement. Don’t use the same volume for reference and deformed unless you have no choice, as you can run into issues where high noise or clipped pixels will not be factored into the error analysis since the program doesn’t have two sets with changing noise pixels to compare.*
4. Run DVC, comparing the reference and deformed volumes
5. Once the DVC run is complete, process the displacements for each imaging setting or DVC setting variation using the DVC analysis protocol, and run *process\_results\_correlationErrorStats3Strain\_XXX.m* to summarize the displacement and strain errors.
6. Refer to the summary variable aSummaryALL to see the bias and absolute average error for each iteration of the image processing settings. Copy the vector into excel to compare settings side by side.
7. Generate a curve showing the variation of the bias and absolute average error in the displacement with the image processing settings to justify your choice of settings. The most important metric to consider is the raw error fields (before thresholding by the error threshold, which will remove the influence of the worst areas). You want to select settings which maximize the correlation area and minimize the error within these areas.   
     
   Notes: Only the displacement errors and correlation area matter for this analysis as we are focused on improving the DVC correlation accuracy. Though we applied a constant strain, the accuracy of strain calculation requires other assumptions and processing, so I find it best to focus on improving the absolute displacement errors and bias and correlation area when I optimize my imaging settings. Essentially, we are trying to find contrast enhancement settings and DVC convergence settings, which allow us to get the most accurate DVC calculations and allow the most areas to correlate. I look at both the raw average errors and the errors after filtering out all error above the error threshold. This allows me to see improvements that might be occurring mainly in the worst areas that may be removed by the error threshold. To calculate average raw error, simply calculate the nanmean() of the uun, vvn, and wwn displacement variables. Absolute raw error is calculated as the nanmean of the absolute value of the uun, vvn, and wwn displacement variables. Raw error has the additional advantage that the domain of averaging is always the same between everything being compared, whereas filtered error may be more similar between iterations since the bad areas are removed prior to averaging. Below are some examples of the kinds of image processing settings that might be optimized:   
     
   - Noise and Background Removal: For LSM images we import images into Huygens Essentials, enter the imaging settings and resolutions, and apply a theoretical deconvolution. For this process, there are 2 user-inputted values that may need optimization: the background value (average guess of the intensity in the background) and the signal to noise ratio. These will affect how well the program removes noise and how well it segments the signal from the background intensity and removes it. You can often eyeball these settings by looking at the results to find values close to optimal to narrow down the range you need to test. 0.5 is the minimum SNR and is what we use for the mouse astrocytes. If you think the SNR is 0.5 or below just use 0.5. Background value should never be over 10 for LSM images as your background should be dark if your images are good. I recommend finding what the background intensity is on average across many runs and using the same value for each image set to be systematic. In previous papers we also removed noise by applying a 3x3x3 pixel median filter. The downside of this is that you blur features in the 1-3 pixel range, so while it works great for suppressing pixel-level noise it has a cost.   
     
   - CLAHE: contrast limited adapative histogram equalization has proved to be effective in increasing contrast and equalizing brightness changes in all image sets studied to date. 2 settings can be optimized for each kind of image: The CLAHE blocksize (local window over which histogram equalization is conducted) and the CLAHE slope (can be thought of as the strength of the contrast enhancement filter, larger values sharpen more, which may not be ideal). These settings should be adjusted to accommodate the size of the features of interest and the amount of contrast variation within them. The optimal settings for OCT images were very different than those used for LSM images for instance.   
     
   - Gamma Shift: for the OCT images, there is not yet a theoretical deconvolution offered. Our features were also very small and were obscured by median filtering, so I instead skipped the noise removal step and directly applied CLAHE. This greatly increased the brightness of the noise and background in addition to the real features. I found that the noise and background could still be suppressed by applying a gamma shift to the image histogram, tuned to make dimmer features dimmer and retain the brightness in brighter areas, and this significantly improved the correlation accuracy as it helped DVC distinguish between background and tissue. Optimized settings for CLAHE can be viewed in the included FIJI script:   
   “CLAHE-STACK-ENHANCEMENT-enhanceALC.ijm”  
     
   - DVC Settings: Optimizing the internal parameters for DVC convergence is conducted the same way you would optimize contrast enhancement settings. The settings you may be interested in optimizing are the starting subset size and ending subset size. These can be edited in the checkConvergenceSSD.m function and the exampleRunFile.m function. You can also set the final calculation spacing or the spacing refinement here; however, I generally don’t optimize this. Spacing affects the amount of calculation points so comparing coarse to fine displacement calculation fields is comparing apples to oranges when it comes to average error. The convergence protocol DVC uses should not need to be edited. Right now I let it iterate, halving the spacing/subset size until it reaches the final size/spacing dictated by the maxIterations variable. If you wish to converge to a calculation spacing below 4x4x2 pixels, then you will need more than 5 iterations. I used 6 for the OCT images.   
     
     
     
   *Figure 2*, raw average and absolute displacement errors evaluated for different gamma shift values in FIJI. Note that the incidence of high error areas decreases significantly for most raw error components as a gamma shift is applied, but not for filtered error components. This is due to the muted background and noise allowing for better segmentation of the tissue from the background and thus less displacement outliers in dim or dark areas or near edges. However, if you only looked at filtered error, these error spots would be removed and you wouldn’t know the correlation was improving in these areas. This is an example of why it is important to look at both raw and filtered error, and also the correlation area, to see if more areas correlated at particular settings. You want as many areas to correlate as possible, as opposed to giving up on them and removing them (filtered error). In this example, a Gamma Shift of 1.75 was chosen as error fell only slightly for higher values and the gamma shift started to cause featured areas to be too dark and to correlate less with much higher values. Though this did decrease average error further, it did so at the loss of correlation.   
     
     
    *Figure 3*, raw average and absolute displacement errors evaluated for different blocksizes used for CLAHE enhancement. Note that the blocksize corresponds to the size of the feature you want to enhance. A smaller blocksize enhances smaller features more effectively, resulting in a sharper look. This is only desirable if these small variations in intensity are real. In noisy images with a lot of variation, a larger blocksize might result in a more accurate correlation. You may also find it useful to only average error in the area you are most interested in. In this case of OCT images in the ONH, I focused on the correlation accuracy of signal within the ONH, which varied only subtly. Because features and contrast variation were small in this area, we used a much smaller blocksize of 14 with a high slope of 3.5 to enhance the signal (CLAHE slope study not shown).

**Protocol: Optimizing Displacement Processing Settings and Strain Calculations**Description: Once the images are obtained and the DVC correlation optimized, then we want to determine what the best way to process the displacements is so that we have the lowest average displacement and strain errors. For this analysis the strain errors are generally the most important metric for optimization; however, displacement errors are still useful to consult and can be improved by some settings such as the correlation coefficient and error thresholds. Other settings (such as what strain calculation routine to call), are more arbitrary and will depend on what you are trying to measure. I generally revised the strain calculation routines for every large study, as what was most appropriate varied with the tissue structure and the image quality. You will want to start with an average image set and conduct an error analysis using an applied numerical displacement and strain (same as previous protocol). It is best to also compare the error fields visually to determine subtle changes in correlation area and to identify and address displacement outliers.

The protocol for optimizing displacement post-processing and strain calculation is as follows:

1. Start with two duplicate imaged volumes (see previous protocol)
2. Enhance both image stacks using your desired image processing protocol
3. Run the virtual\_displacement.m matlab file, and enter your first and second volumes as the reference and warped volumes to process in the file. This will apply a numerical displacement, simulating an RBM and constant strain, to the volumes and rename them (see previous protocol)
4. Run DVC, comparing the reference and deformed volumes
5. Start with the DVC correlation results file and process the displacements using the DVC analysis protocol and the individual process\_results file. In this file you can vary to parameter cc\_threshold to edit the correlation coefficient threshold. This is the first of the parameters you want to optimize. You want to find a value that segments the tissues from the background. Displacement error is the best metric to consult when optimizing this. Only vary one parameter at a time when optimizing, starting with the earlier settings.
6. Then run *process\_results\_correlationErrorStats3Strain\_XXX.m* to summarize the displacement and strain errors. Here you can vary the error threshold value (I generally set it to approximately one pixel in the smallest dimension), the size of the Gaussian smoothing filter, and the strain calculation routine you call. In the past I’ve used many different strain methods and you may need to similarly develop your own.
7. Refer to the summary variable aSummaryALL to see the bias and absolute average displacement strain error for each iteration of the displacement post-processing settings. Copy the vector into excel to compare settings.
8. Generate figures of the displacement and strain fields and overlay the max-projected original images on them in powerpoint. Flip between slides to compare how correlation area and error varies as you vary settings. Choose settings which both minimize average error and increase correlation area  
      
   Notes: This kind of optimization is less straightforward than the previous protocols. I usually worked in powerpoint and generated plots of the Z-projected error. I then overlaid the Z-projected images to see how the fields correspond to structure as this helped me choose a good correlation coefficient threshold. You can then flip between slides to see how the error field and correlation error vary as you vary settings. I recommend starting with optimizing the earliest parameters used and then moving on to other ones used later in the analysis. A good order is: correlation coefficient threshold, error threshold, deviation outlier filter, smoothing, and then strain calculation. When it comes to the best choice of smoothing and strain calculation and their domain selection, there isn’t a clear-cut answer and it can take some investigation. The above methods can also be used to evaluate any new post-processing function you want to develop or test if you think it will be helpful in addressing a systemic issue (such as poorly calculated strains in a particular region). For the clinical OCT image analysis, several new functions or methods were developed and tested in this fashion. Below is a list of the commonly used displacement processing methods and how they may be optimized:   
     
   **- Correlation Coefficient Threshold:** The only parameter in the individual process\_results file that must be optimized. Once a value is chosen, try to use it for every image set. The only exception is if you have no way to do noise removal. In these cases, the background intensity varies from image to image (this was the case with OCT) and the value must be varied a bit to be optimal for each eye. This should hopefully not be the case with your images, and it is preferential to only vary it and choose the value once after an error study. Use an average eye instead of a good or bad one to get the best average value. You will notice the higher this parameter, the more areas get marked as background and removed. A good value will only remove areas corresponding to black space or indistinct background. You will find almost all displacement outliers (sharp inaccurate discontinuities) appear in indistinct areas such as background and a well-selected threshold will remove most of these problem areas.   
     
   - **Error Threshold:** The displacement error threshold (in microns) above and below which a DVC displacement calculation is flagged as poorly correlating and removed. This setting is best run as a follow-up after correlation coefficient filtering, which is good at segmenting background but not at identifying and removing bright but featureless areas. The error threshold should be set to remove calculations that have an error of about a pixel or more. Don’t bother optimizing it, as you will just find the lower the threshold the better. Due to the presence of creep in our images, I find setting it lower then 1 pixel can sometimes mean large areas get removed due to creep. Whatever you choose in the future, at the very least it should be set a bit higher then the expected creep magnitude.   
     
   **- Deviation Filter:** After correlation coefficient and error filtering small isolated displacement outliers may still remain in the displacement fields. These can influence the strain calculations, so I often apply a deviation filter to identify and remove un-physiological spikes in the displacement field. You can vary the size of the area searched and the deviation amount used to detect displacement outliers in the function Deviation\_Filter.m. Larger sizes are more appropriate for larger error spots. The filter will often not remove error spots that are about half the size of the filter or more, as the spot will bias the surrounding average. The idea is that the error threshold and correlation coefficient thresholds will remove large areas with systematic error and that this filter only has to find small isolated spots that are left on the edges (see Dan Midgett’s thesis, Pages 200-201).  
     
   - **Gaussian Smoothing Filter:** This filter smooths the displacements with a moving 3D Gaussian kernel so that the average of the surrounding displacement field and all large displacement gradients are preserved. It will also interpolate small holes left in the displacement field from error removal, and these areas will get reinterpolated. The main thing you need to optimize is the size of the filter. Internally, you can also vary the width of the standard deviation (sigma) of the distribution if you want more or less aggressive smoothing. I generally try to have at least one Gaussian standard deviation within the box size I am using. What you choose will depend on what you want. Keep in mind that stronger or larger filters will usually result in more accurate average strain calculations, but this may not be ideal if for instance you intend to study small regional variations in strain. The filter size should be no larger than about half of the size of regions you want to compare locally in this case. Since there isn’t a good way to optimize this via minimization, I usually just look at the strain error fields in powerpoint and increase the Gaussian filter size until small displacement error spots (small areas with error under the error threshold) are smoothed out and don’t affect the average strain calculations much.   
     
   - **Strain Calculation Method:** This can vary. I’ve developed several methods and I found the most accurate ones for this data to be global polynomial fitting (for a continuous area) or fitting locally over a square region centered on each point (for more irregular areas). There are several variations of these methods I’ve used as well. In the case of the polynomial the only thing you need to optimize is the fit order. In the case of the local strain calculation method, the thing you need to optimize is the box size. For custom strain calculation methods you develop in the future, you can optimize their settings using this same protocol.