HOW TO - Measure mean speckle width on in-focus images of speckles

1. Make a homemade fluorescent slide if needed :
    1. Clean up a microscopic slide with a kimwip and methanol. Let it dry for at least one minute.
    2. With a 20 uL micropipette, drop 20 uL of homemade fluorescent solution (liquid FITC in distil water for 488 excitaiton laser) off on the microscopic slide.
    3. Cover the drop of fluorescent solution with a cover slip.
    4. Seal the cover slip on the microscopic slide with nail polish. Let it dry five minutes.
2. Find the in-focus plane of speckles using the fluorescent slide. Tip : The in-focus plane can be hard to find and to see using a standard acquisition software. A good way to find it is to look at the image's fast Fourier Transform (FFT); the plane where there are more high frequencies is the in-focus plane.

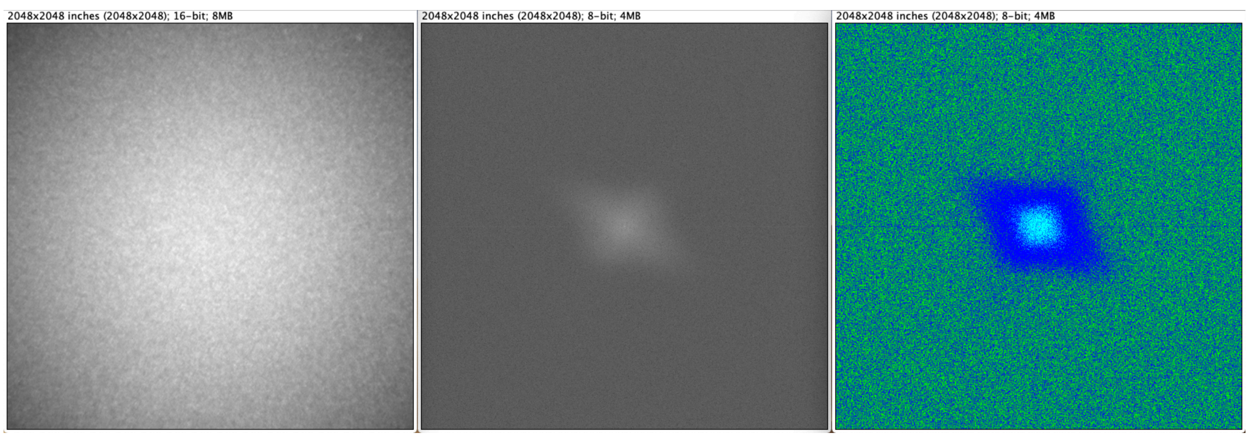

Figure 1 : Raw image of in-focus speckles (left), FFT of this raw image (middle) and the same FFT image with brgbcmyw colours (right) on Fiji.

3. If the in-focus plane is hard to distinguish, acquire a z-stack of a couple of microns around the in-focus speckles plane to make sure to acquire the plane where the speckles are perfectly in focus. Otherwise, acquire the in-focus image of speckles.
4. Measure the mean speckle width of the in-focus image on Fiji :
    1. Open this image in Fiji and select it.
    2. Process > FFT > FFT.
    3. Crtl+a to select all the FFT image.
    4. Ctrl+k to show the *plot profile* of the FFT image.
    5. Extract points of this *plot profile* by clicking on *List* in the *Plot Profile* window.
    6. Delete from this list of points the ones that form the huge peak in the middle of the gaussian curve. It might be one or multiple points : Select those points and right click on the mouse, *Delete*.
    7. Select and copy all the points with right click on the mouse, *Select All* and *Copy*.

8. Analyze > Tools > Curve Fitting... Paste the list in the box of the window named *Curve Fitter*.
9. Choose *Gaussian* as the type of curve to fit the list of points. Make sure that the *Show Settings* box is checked before clicking on the *Fit* button.

5. Calculate the FWHM with the python code *FWHM calculation with Gaussian Fitting.py* found on GitHub > DCC-Lab > HiLoZebrafish > FWHM_GaussianFittingCurve :

    1. In the *Log* window on Fiji, the values of the *a, b, c* and *d* parameters are written for the present curve fitting. Match those values to the appropriate variables in the python code (*a* in Fiji goes with *a* in the python code, etc.).
    2. Run the python code.
    3. Values of the *a, b, c* and *d* parameters, roots of the gaussian function and FWHM are printed in the terminal. Those datas can be saved in a excel file, for example.

6. Measure the mean speckle width using the FWHM :

    1. Draw a line on the FFT image from the middle to the right with the total lenght of the FWHM calculated previously.

    2. While doing the line, look at the Fiji window ; a value of pixels per cycle appears according to the position of the cursor in the FFT image. When the line with the lenght of the FWHM is drawn, look at this value of pixels per cycle. It corresponds to the half mean radius of speckles in the raw image.

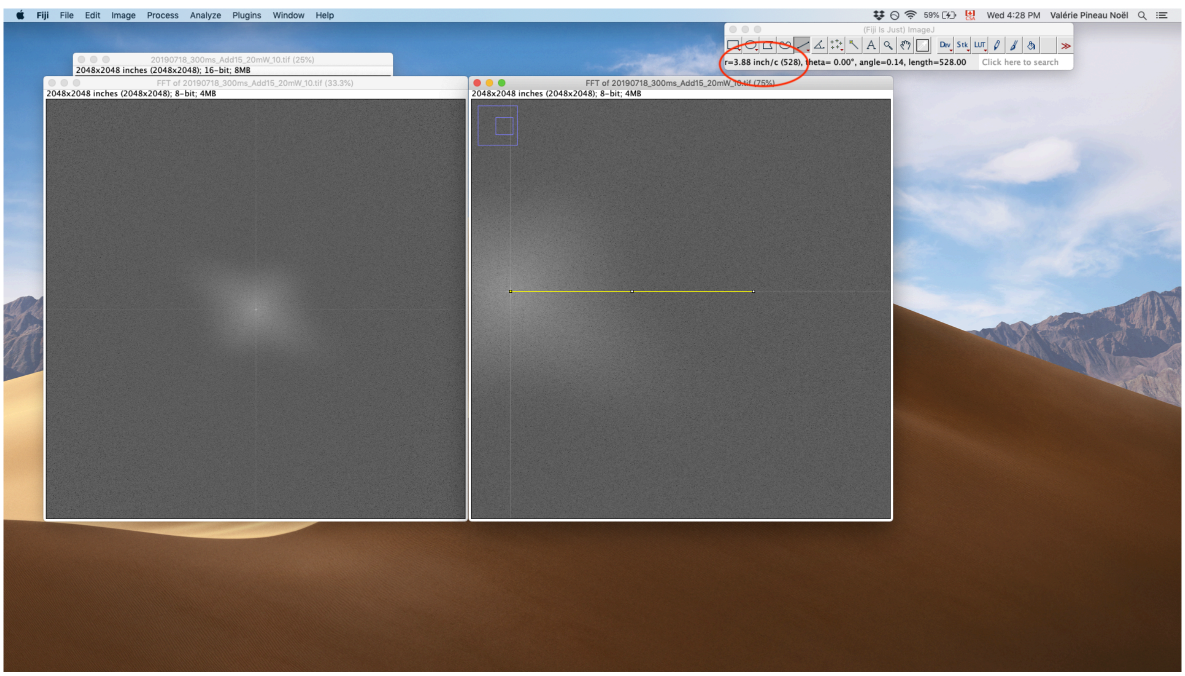

Figure 2 : Printscreen of the line on the FFt image. On the left, the FFT image on a raw image on in-focus speckles; on the right, a zoomed part of the FFT image. The line has the same lenght of the FWHM calculated previously. The value encircled in red is the number of pixels corresponding to this FWHM value.}

    3. Save this value in an excel table, for example.

7. Calculate the corresponding mean speckle width...

    1. ...in pixels :

$$W_{speckles} = R_{speckles} * 4$$

Where $W_{speckles}$ is the mean speckle width in pixels and $R_{speckles}$ is the half mean radius.

2. ...in microns :
    1. You could use the pixel width of the camera found in the datasheet. Otherwise, you could use an experimental measure made with known width beads larger than the lateral resolution.
    2. $W_{microns} = \frac{Number\ of\ pixels}{Number\ of\ micron\ (\mu m)} * W_{speckles}$

    Where $W_{microns}$ is the mean speckle width in microns.