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Fluorescent foci quantitation for high---throughput analysis

Elena Ledesma-Fernández and Peter H. Thorpe

Supplementary Information

Contents

Supplementary Text

ImageJ script for quantitation.
The script is split into modules each of which is described separately here.

1. Openfile

The user is asked to select a folder that contains microscope images. The folder should not contain other files. The module uses ImageJ's Bioformats function to open image files. Bioformats works on many different microscope file formats including the OME-TIFF format. However, each file should be a separate image. If single files contain more than one image, then the script will fail. The module takes the first channel (others are ignored) and saves two maximum projections images of the stack. The first is for identifying the x-y position of foci the second is for the labels. The module also saves an average projection of the image.

2. Select Coordinates

There are three different versions of the script each of which uses a different method to select the foci.

a) Manual selection. A maximum projection image is opened and the user should select each focus using the point selection tool. Holding down the alt or shift key allows for multiple foci to be selected. The resulting selection coordinates are stored in two arrays (x and y).
b) Semi-automated selection. A maximum projection image is opened and the user should read the instructions and press 'OK'. The FindMaxima window appears and by selecting 'Preview point selection' the selected foci can be viewed. Next the value for 'Noise tolerance' can be adjusted higher for a more stringent selection or lower for a more relaxed selection. Once an appropriate selection is achieved the user clicks 'OK' and the resulting selection coordinates are stored in two arrays (x and y).
c) Automated selection. The FindMaxima script is run automatically using the noise values specified at the start of the script. The output is a set of selection coordinates, which are stored in two arrays (x and y).

3. Quantitation

Each x-y coordinate from the selection of foci is used as the centre of a local search area in the maximum projection image to reidentify each focus. The size of the search area can be altered but should contain only one focus. The output of this is the final position of the focus, reported as X and Y in the results table. The script then calls four successive modules to assess each focus; Checkz, quantifyKinetochores, quantifyBackground and labelKinetochores.

4. Checkz

The checkz module extracts from the original microscope image a small square centered upon the focus. The brightest slice within this stack is determined using a 'searchslice' module (described below), this will define the z slice used for quantitation (listed in the results table as Z). If the brightest slice is bleached or if the z slice is at the very bottom or top of the image stack then an error is returned and no further quantitation is performed.

5. QuantifyKinetochores

The quantifyKinetochores module extracts from the original microscope image a small square centered upon the focus and repeats the searchslice module to identify the brightest Z slice. This brightest z slice is selected and quantified. Then the "measurebox" module is used to measure the z slices immediately above and below the brightest slice (described separately). The average projection image is opened and the average kinetochore measurement is made.

6. QuantifyBackground

The quantifyBackground module measures mean and median of an area surrounding the measured kinetochore (as illustrated in Fig. 2C) from the original microscope image in the z slice corresponding to that measured for the kinetochore. The same area is also measured (mean) from the average projection. The Findxy module is called from here also to precisely define the position of the focus (described below).

7. LabelKinetochores

The labelKinetochores module adds labels to a maximum projection image. For each kinetochore these include a red box surrounding the measured kinetochore area and a white outline of the background area, finally a text label defining the kinetochore number identified in the image. This number can be used to cross reference with the tabulated output data to identify values for a specific kinetochore in an image. An example of the resulting labels is shown in Figure 2E. For the two-colour scripts a small yellow square indicates the centre of the foci that was identified in the second fluorescence channel, as shown in Figure 6D.

8. SearchSlice

The seachSlice module iterates through every z slice in a stack of images and checks where the maximum pixel value is bleached (for 12-, 14-, 16- and 32- bit encoded images). The slice with the maximum intensity is returned.

9. Measurebox

The measurebox module measures the mean value of each kinetochore area in three different z slices; these include the brightest slice and the one immediately above or below the brightest slice. The average of these is returned as the "Box Mean".

10. Findxy

The findxy module records both the centre of mass of the fluorescence in a region that is 2 pixels larger than the measured kinetochore region. This same larger area is used to plot Gausian curves of intensity in first the x and then the y dimension. Briefly, for the x dimension the sum of intensities in each column is used as a point on a curve and the "Fit.doFit" ImageJ function is used to fit a Gaussian function to these data. The only data extracted from the curve fitting the peak position. In the Y dimension the sum of the intensities in each row is used to derive the data for curve fitting.

Volocity Protocol

To compare our quantitative analysis with that produced from a commercial software package we made use of the Volocity image analysis software from Perkin Elmer (USA). A script was written for the Volocity quantitation module that automatically detects foci and quantifies them. The steps used are defined below:

1. The "FindSpots" function was used to identify bright spots in the YFP channel.
2. The kinetochore volume was defined as this single voxel (a 3D pixel) dilated three times. "Dilation" extends the volume by a single pixel in all dimensions.
3. The background volume was defined as by the single voxel dilated 5 times, subtracted from the single voxel dilated 8 times.

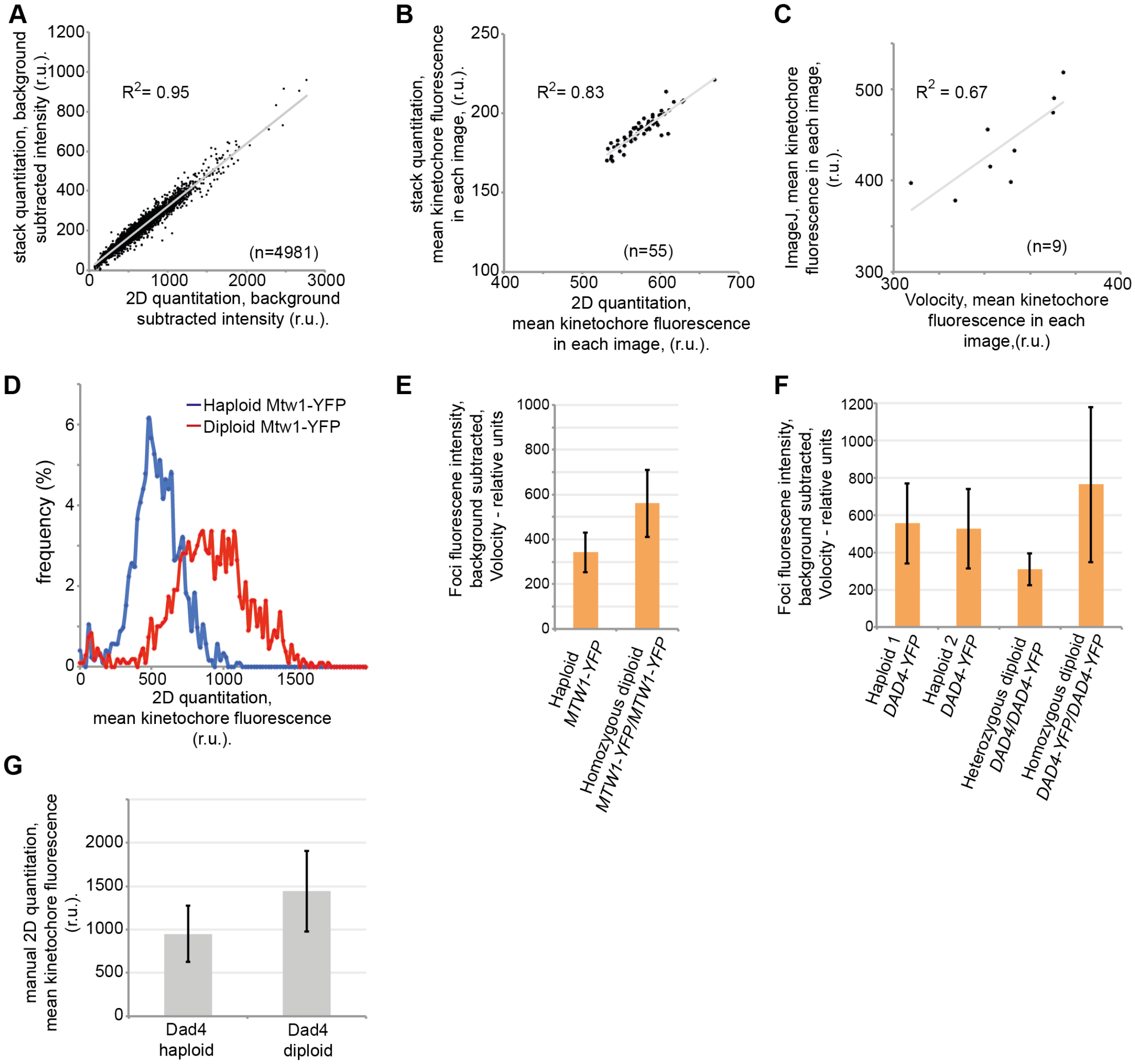

Figure S1. Comparison of quantitation methods for fluorescent foci. A. The measurement of fluorescence from the entire stack of z slices is compared with the 2D measurement for 4981 Dad4-YFP foci. **B.** The average foci intensity of each image also correlate well when the whole stack method is compared with the 2D measurements. **C.** The mean fluorescence intensities of the 3D kinetochore measurements in each image with the automated ImageJ script (y axis) are compared with the 3D measurements using Volocity software (x axis), for nine separate images. **D.** The distribution of intensities of Mtw1-YFP foci from haploid cells (blue) and diploids (red) are shown; the measurements were made using the automated 2D method. **E.** The mean fluorescence intensity of Mtw1-YFP foci in both haploids and diploids is shown for Volocity automated analysis, the error bars indicate +/- one standard deviation. **F.** The mean fluorescence intensity of Dad4-YFP foci in two independent haploids, a diploid and a heterozygously-tagged strain is shown for Volocity automated analysis, the error bars indicate the standard deviation **G.** The intensities of Dad4-YFP foci in haploid and diploid cells are compared using the manual 2D method. The diploid mean intensity is 52% higher than the haploid and the error bars shown the standard deviation, the coefficient of variation is 0.34 for haploids, 0.32 for diploids.

	Label	Area	Mean	StdDev	Min	Max	X	Y	XM	YM	Median	Z	Box Mean	Z Stack Mean	3D Sum	X-Gauss	Y-Gauss
1	IP-test-03-Scene-1-P10-01--kinetochores 1	0.377	2869	2961.098	520	7899	7.943	192.581	8.04	192.517	781	6	1269.444	581.667	21530	8.048	192.515
2	IP-test-03-Scene-1-P10-01--background 1	3.019	470.792	14.274	436	507	7.883	191.145	0	0	472	6	0	472.639	0	0	0

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47	IP-test-03-Scene-1-P10-01--kinetochores 24	0.377	1100.444	403.007	687	1921	67.563	65.913	67.54	65.865	968	15	1054	699.444	14202	67.548	65.85
48	IP-test-03-Scene-1-P10-01--background 24	3.019	528.417	22.697	482	602	67.06	65.421	0	0	528	15	0	538.389	0	0	0
49	IP-test-03-Scene-1-P10-01--kinetochores 25	0.377	1363.333	236.185	1083	1832	6.086	95.826	6.136	95.818	1371	12	1318.037	816.444	21520	6.21	95.824
50	IP-test-03-Scene-1-P10-01--background 25	3.019	534.236	58.948	459	790	6.04	95.112	0	0	521	12	0	549.931	0	0	0
51	IP-test-03-Scene-1-P10-01--kinetochores 26	0.377	1096.556	431.984	617	1898	112.743	37.031	112.777	37.022	926	15	1067.333	705.111	148051	112.819	37.01
52	IP-test-03-Scene-1-P10-01--background 26	3.019	520.333	20.326	479	592	111.902	36.755	0	0	519	15	0	527.111	0	0	0

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Table S1. Output tabulated data from FociQuant. A selection of the tabulated data from the automated measurement of foci from the image shown in Figure 2A is shown. For each focus region and each background region there is a separate line of data. The label for each line indicates the image name (in this case "IP-test-O3-Scene-1-P10-01") appended with an identifier of either "kinetochore" or "background" followed by a number which indicates which focus is being measured – this focus number is included in the output image labels. The table is truncated after the first measurement to allow the inclusion of kinetochore number 25, which is shown in detail in Figure 2. The kinetochore intensity measurements include the "Mean" and "Median" as well as the "Box Mean" and "Z Stack Mean". The background intensity measurements included "Mean", "Median" and "Z Stack Mean". Additionally, the data include the "Area" of the 2D measurement, the "StdDev" (standard deviation) of the mean measurement, the "Min" and "Max" pixel values for the 2D measurement. The "X", "Y" and "Z" values indicate the pixel position of the foci in 3 dimensions. The "XM" and "YM" values indicate the centre of mass of the foci. "3D Sum" is the integrated 3 dimensional fluorescence measurement and the "X-Gaus" and "Y-Gaus" indicate the centre of the Gausian plot of each foci. In all cases the X and Y positions are in µm, whereas the Z stack position indicates the z slice that was used as the centre of quantitation for each focus.

	Label	Area	Mean	StdDev	Min	Max	X	Y	XM	YM	Median	Z	Box Mean	Z Stack Mean	3D Sum	X-Gauss	Y-Gauss
Table truncated here																	
669	YR(146-1A)-Scene-05-P7--kinetochores 16	0.383	1484.556	325.562	1005	2024	122.852	63.437	122.876	63.433	1349	18	1449.444	1035.111	39135	122.905	63.477
670	YR(146-1A)-Scene-05-P7--background 16	3.066	807.667	98.423	715	1279	122.881	63.452	0	0	776	18	0	820.931	0	0	0
671	YR(146-1A)-Scene-05-P7--2nd channel of foci 16	0.383	1363.556	270.342	1083	1900	122.645	63.644	122.719	63.749	1312	15	1319.333	1019.667	35622	fail	63.693
672	YR(146-1A)-Scene-05-P7--ch2 background of 16	3.066	900.417	124.816	795	1450	122.675	63.659	0	0	866	15	0	883.583	0	0	0
Table truncated here																	
705	YR(146-1A)-Scene-05-P7--kinetochores 25	0.383	1309.444	201.684	1016	1693	123.264	62.818	123.221	62.875	1277	17	1301.63	974.111	35144	123.185	62.919
706	YR(146-1A)-Scene-05-P7--background 25	3.066	831.028	177.52	696	1799	123.294	62.833	0	0	789	17	0	828.917	0	0	0
707	YR(146-1A)-Scene-05-P7--2nd channel of foci 25	0.383	1164.333	192.874	915	1450	123.264	62.612	123.325	62.773	1158	15	1138.926	943.222	30751	fail	62.713
708	YR(146-1A)-Scene-05-P7--ch2 background of 25	3.066	897.681	162.476	782	1900	123.294	62.627	0	0	858	15	0	877.403	0	0	0
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Table S2. **Output tabulated data from 2 colour FociQuant**. A selection of the tabulated data from the automated measurement of foci from the image shown in Figure 6D is shown. The format of the table is the same as Table S1, except for each foci there is an additional measurement of the foci in the second channel (red in this case). The table is truncated to allow the inclusion of the two kinetochore foci in Figure 6D (‘kinetochores 16’ is the top focus and ‘kinetochores 25’ is the bottom focus in Fig. 6D).