## The following protocol and information has been adapted from the PhD thesis of Joe Weber (10.2020). Last update JW 12.2020.

## Protocol for chromosome segregation analyses (DNA content and FISH)

1. Prepare microscopy slides with fixed squashed testes with DNA stain (see White-Cooper, 2004) according to protocol 3.3.2. Optional: perform FISH staining as previously described (Sun et al., 2019).
2. Take 40x image stacks of post meiotic cysts with early round spermatids with 280 nm z-spacing. Image 10-20 cysts per genotype. For XY FISH analyses the images must have 3 channels in the following order: “blue”, “green”, “red”. For autosomal FISH analyses the images must have 2 channels in the following order: “blue”, “red”. For DNA content analyses the number of channels doesn’t matter. However, if the DNA channel is not the first channel line 4 of the DNA content analyses.txt script has to be manually changed.
3. Click on the icon with the I to run the “Identify nuclei” macro and follow the instructions. Delete ROIs on cyst nuclei when prompted to do so. If two overlapping nuclei are recognized as one also delete this ROI. If FISH analyzes is intended draw the correct ROIs manually and press t to add to the ROI manager.
4. Repeat step 4 until all images have been processed.
5. Run the “merge DNA content results” python script and follow the instructions to add results from multiple genotypes.
6. Run the “process DNA content results” python script.
7. Run the “scatterplot DNA content results” python script. Review the data on the plot. Genotypes with wildtype chromosome segregation have around 0.1 average standard deviation. Genotypes with random chromosome segregation in meiosis I have around 0.42 average standard deviation. Save the plot as .svg and/or .png.
8. To better compare genotypes in one plot run the “barplot DNA content results” python script. Save the plot as .svg and/or .png.

The following steps are only needed if FISH signals are analyzed

1. Load the toolset “XY FISH analyses” or “autosomal FISH analyses”
2. Click on the icon with the F to run the “Identify … FISH signals” macro and follow the instructions.
3. Look at each nucleus and make sure the signals have been identified correctly. To correct eventual mistakes, use the tools with the colored circle icons. For XY FISH: blue is for no signal, green is for Y only, red is for X only and white is for X and Y. For autosomal FISH: blue is for no signal, green is for 1 signal, red is for 2 signals.
4. When all nuclei have been assigned the correct color click the “Done” icon to run the “confirm … FISH ROIs” macro.
5. Run the “merge … FISH results” python script and follow the instructions to add results from multiple genotypes.
6. Run the “barplot FISH results” python script to obtain a barplot comparing the different genotypes. Save the plot as .svg and/or .png.

## Details

### Requirements to run the DNA content analyses pipeline

The DNA content analysis pipeline consists of three ImageJ macros and four python scripts (Fig 3.1C). To use the ImageJ macros, a functional installation of FIJI (Schindelin et al., 2012) is required*.* The ImageJ macros are packed in the form of an ImageJ toolset. Macros in a toolset can be conveniently started from the toolbar in ImageJ (Fig 3.1B). To install a toolset in FIJI, a .txt file containing the code must be placed in “C:\Users\username\Fiji.app\macros\toolsets”. To use the python scripts, python 3.7 or later is required. Furthermore, four python packages need to be installed: “NumPy” and “pandas” are required for scientific computing, “Matplotlib” and “Seaborn” are required for data visualization.

### Slide preparation

Testis squash preparations should be prepared essentially as described previously (White-Cooper 2004; protocol 3.3.2). In brief, 5-10 pairs of testes are dissected from 1-3 days old males. On a poly-L-lysine-treated slide, the testes are cut open to release their content. Early postmeiotic cysts with round nuclei at the onion stage are generally found at about 1/3 of the length of the testis tube. Therefore, I recommend focusing on releasing cysts from this region. The testes are then gently squashed under a coverslip to spread the released cysts. After transient quick freezing in liquid nitrogen, testes are fixed using formaldehyde and stained for DNA using Hoechst 33258. The DNA content analyses can be performed in parallel with FISH, in that case the FISH staining should be performed as described in (Sun et al., 2019).

### Imaging

The image analyses pipeline has been set up using 1388x1040 pixels images from a Zeiss Cell Observer HS microscope with a 40×/0.75 oil immersion objective. The z-step size of the images was 280 nm and the number of slices was chosen in a way that all the nuclei from the cyst to be analyzed are fully included (usually 30-50 slices). Cysts of early post meiotic cysts with round nuclei at the onion stage are optimal to measure the DNA content. The hybridization process of FISH staining often results in fuzzy and more spread out DNA staining. The DNA content analyses is robust and works even with DNA staining of relatively low quality. However, if the difference in signal intensity between the nuclei and the background is too small, the recognition of nuclei fails. If more than 10 percent of the nuclei are not recognized the image should be discarded.