Analyzing gamma H2AX foci

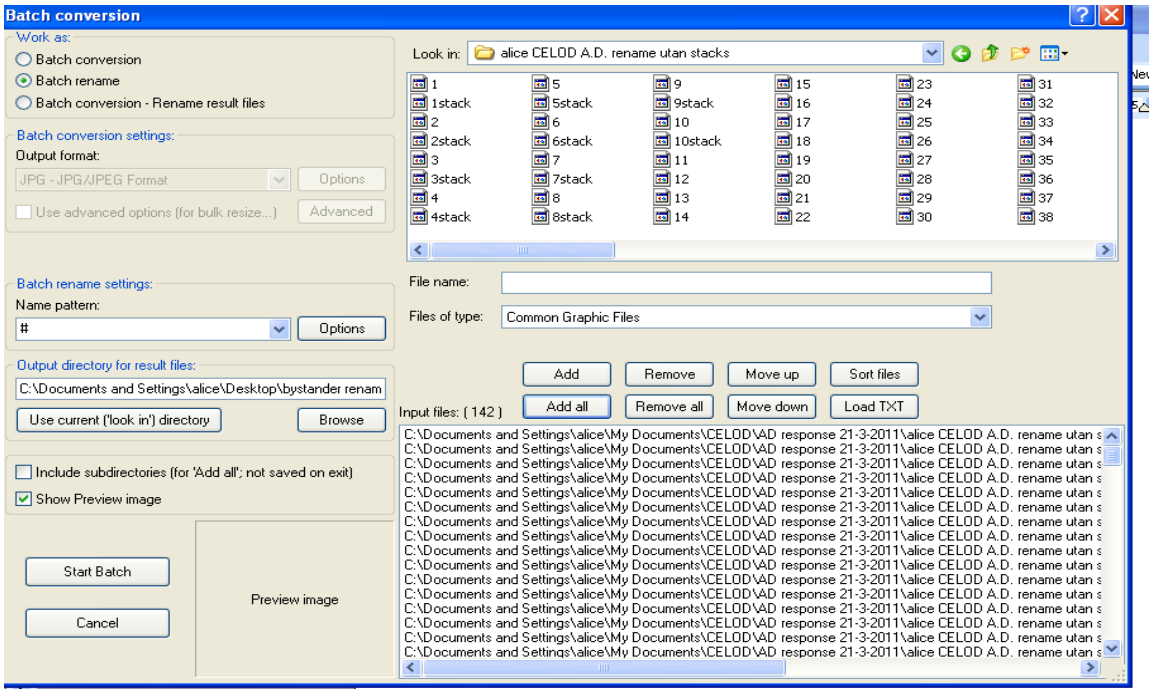
The following items are needed for analysis, and you find the links on the CELOD home page:

File renaming programs:
IrfanView (for PC): https://www.irfanview.com/
Namechanger (for Mac): https://namechanger.en.softonic.com/mac

The image analysis software used to analyse foci:
ImageJ v 1.43u, together with the macro - **macro gammaH2AX foci.txt**
*The newer Fiji ImageJ version will also give you data output if you use this macro version (**macro gammaH2AX foci for Fiji**, where the text line coloc is replaced with cool). However, it does not display the foci in the stacked images, i.e. using the newer ImageJ versions you cannot do the quality control of the threshold level of foci detection.*

Images in TIF format (containing layers for each individual colour):
Image galleries - GammaH2AX images for scoring (ZIP file)

IrfanView
1) To **rename the pictures** do as follows:
Create a new folder on the desktop. Name it like the dose you want to analyse plus rename. Ex "Control rename". This is the folder that you will give as "output directory for result files". In other words all the renamed pictures will be saved here.
Now **open IrfanView**.
File → Batch conversion/Rename → Work as: Batch rename → Batch rename settings: name pattern: #
Output directory for results files: Browse → (open the folder previously labelled "rename" ex. Control rename) → OK
Look in: (open the folder with your original image files for control)
→ Add all.
You will see this on your computer:



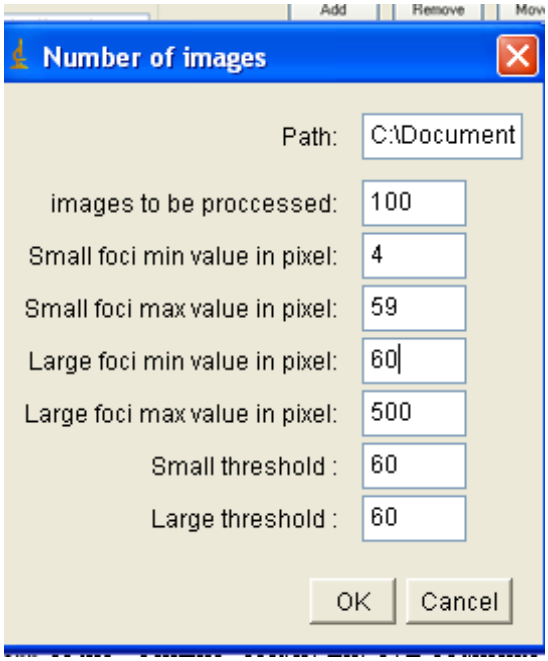
And finally **Start Batch**

Now all the original pictures have been transferred to the “rename” folder and they will be numbered from 1 to ….n. Open the “rename” folder and check that the pictures are renamed in the right way: 1; 2; 3; ….n

Repeat the file renaming for the folders containing images for the other doses. Close IrfanView.

Copy the address of the “rename” folder. Ex: C:\Documents and Settings\alice\Desktop\alice CELOD Control Rename \.

Once you have copied the address, **open ImageJ**
File → Open → (open the **macro gammaH2AX foci.txt**).
In the **macro gammaH2AX foci.txt**: Macros → Run macro



Path: (Paste **the address** of the "rename" folder).
Do **not forget the \ at the end of the address** or the program will not recognise the folder.

Images to be processed: 100
Small foci min value in pixel: 4
Small foci max value in pixel: 59
Large foci min value in pixel: 60
Large foci max value in pixel: 500
Small threshold: 60
Large threshold: 60
Ok

Those settings are indicative but they can be changed depending on the cell line. To verify the results look at the black and white stacks produced in the "rename" folders. Each stack has three pages where cells are numbered and foci are analysed.

- *Images to be processed: Increase in case you want to analyse >100 images simultaneously.*
- *The cutoff foci value in pixels (here 60) determines the separation between large and small foci.*
- *The small and large threshold can be lowered/raised in case the image was taken at a suboptimal acquisition time and not all foci/too many foci are detected.*

Now ImageJ will count the foci.
When the program stops, **open the "Log" file**. Here there are all the results.
Copy all the data: Ctrl+A; Ctrl+C
Then paste them all into an Excel file.

Open a few of the stacked images (1stack.tif, etc. which now have appeared in the rename folder), click between the layers (page 1-3) using the Windows Photo Viewer or any similar program.

- The nuclei are encircled with a blue ring and are all identified by numbers
- Small foci appear as red dots
- Large foci appear as green dots

Insert the headlines in Excel (those in yellow are relevant for the analysis).

Picture no.	Cell no.	DNA area	DNA mean	SF mean	SF sd	LF mean	LF sd	SF area	SF intensity	No. of SF	LF area	LF intensity	No. of LF	Not used	Not used

SF= small foci
LF= large foci

Make an extra column where you calculate the **total number of foci** = No. of SF + No. of LF.

*If pictures were taken in samples with high cell concentration, it can happen that part of a cell is cut off leaving a "half" cell in the picture. Other times two cells can be so close to each other that the program wrongly recognize them as one single cell. The "half" cells will get less foci than the average while the double cells will get more. For reliable results, it is important to delete them from the analysis. The easiest way to do it is to remove the cells with a too small ("half" cell) or too big (double cell) nucleus (**DNA area**).*

To do so, sort data based on DNA area:
Mark all your data. Data → Sort → DNA area. Remove cells a with DNA area smaller than 10 000 and larger than 30 000.

The DNA area values must be adjusted depending on the cell line used. This is done by manual evaluation of the black-and-white stacks produced in the "rename" folders, where all nuclei can be individually identified and correlated to the DNA area in the Excel file based on the image and cell numbers. In addition, it is recommended to seed the cells on cover slips at least 48 h before the fixation since the DNA area depends on how much cells have flattened on the cover slip.

Sort the cells again based on picture number: 1) Mark all data. 2) Data → Sort → Picture number.

For each dose choose 50 cells randomly (ex from row 25 to 75) and calculate:
Average foci number
Average LF number
Average SF number
Average LF intensity (Optional)
Average SF intensity (Optional)

>50 cells can be included in the analysis but it is a way to equalize the variation between samples, because different numbers of cells are present in each photo and have been excluded based on DNA area in each sample.

Formulas in Excel:
For the average: **=AVERAGE( )**
For the standard deviation: **=STDEV( )**

Give your data to the group responsible for the gamma H2AX analysis.

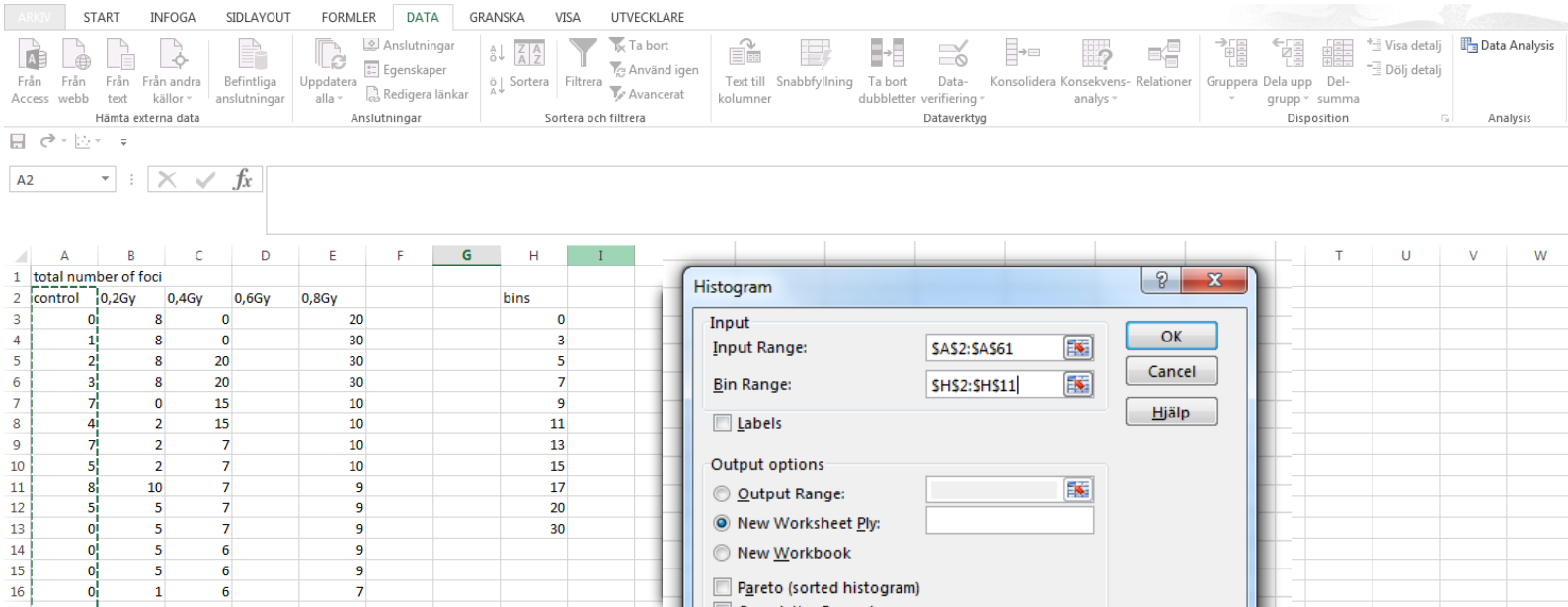
For the group doing the gH2AX presentation:
Per each dose, show the distribution of the total number of foci (in the cells everyone has analysed) by constructing a histogram (a column chart that displays frequency data) using the Histogram tool in Excel.
Using this tool, Excel will count the number of **cells with a certain number of foci (**specified in the bins).

To use Histogram tool you need to install Analysis ToolPak: (https://support.office.com/en-us/article/Load-the-Analysis-ToolPak-in-Excel-6a63e598-cd6d-42e3-9317-6b40ba1a66b4#OfficeVersion=Windows)
To install **Analysis ToolPak:**

a) On the **File** tab, click **Options**.
b) Click **Add-Ins** in the navigation pane.
c) In the **Manage** box, select **Excel Add-ins**, and then click **Go.**
d) In the **Add-Ins** dialog box, make sure that the **Analysis ToolPak** check box under **Add-Ins available** is selected, and then click **OK**.
e) Now you should be able to see Data Analysis in the Data Tab (see the excel sheet)

To construct the histogram, start by organizing your data in **two new columns** in a new sheet. These columns must contain input data and bin numbers. Input data is the data that you want to analyse by using the Histogram tool **(in this case, total number of foci).** Bin numbers are the numbers that represent the intervals that you want the Histogram tool to use for measuring the input data in the data analysis (**in this case**, **number of foci per cell**).
In other words: Per each dose, copy-paste the column with the total number of foci on the new worksheet in one column and write the bin range (upper limit) in another column. Suitable bin range can be: 0;3;5;7;9;11;13;15;17;20; 30. But you can decide your own bin range.

To make a histogram: Data → Data Analysis → Histogram. Input range will be the total number of foci per each dose. And bin range the numbers inserted under bins (foci per cell). Select the option Chart Output.



	A	B	C	D	E	H
1	total number of foci					
2	control	0,2Gy	0,4Gy	0,6Gy	0,8Gy	bins
3	0	8	0		20	0
4	1	8	0		30	3
5	2	8	20		30	5
6	3	8	20		30	7
7	7	0	15		10	9
8	4	2	15		10	11
9	7	2	7		10	13
10	5	2	7		10	15
11	8	10	7		9	17
12	5	5	7		9	20
13	0	5	7		9	30
14	0	5	6		9	
15	0	5	6		9	
16	0	1	6		7	

1) Collect the Average foci number, Average LF number and Average SF number from each member of the group. Calculate the average and standard deviation within the group. Plot it in a bar chart with number of foci per cell as y-axis and dose as x-axis