Hemagglutination Macro for ImageJ v1.0.2

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The intended use of this macro is for screening of lectin activity in raw or cooked dry bean samples and other pulse crops. Interpretation of hemagglutination assay results by eye can be quite subjective with high interobserver variability when attempting to determine positivity based on hemagglutination units (HAU). This macro provides an objective alternative by measuring the area (mm^2) of agglutinated red blood cells in a single well, one row of 12 wells at a time, in 96 well round bottom plates. A region of interest (ROI) set consisting of a 12 well overlay is moved in unison by the user from row to row as the macro loops through analysis until all rows have been read. An Excel template is provided that can be used for data collection and summary.

Companion videos are also provided showing how to perform the hemagglutination assay in a 96 well round bottom plate using defibrinated rabbit red blood cells, creating a 12 well ROI set in ImageJ and using the ImageJ macro for analyzing hemagglutination plate images.

Lectin Hemagglutination Assay
https://www.youtube.com/watch?v=FWOMhOlLiug

Creating a 12 Well ROI Set for Analyzing Hemagglutination Plates in ImageJ
https://www.youtube.com/watch?v=d4BtTiAVIGo

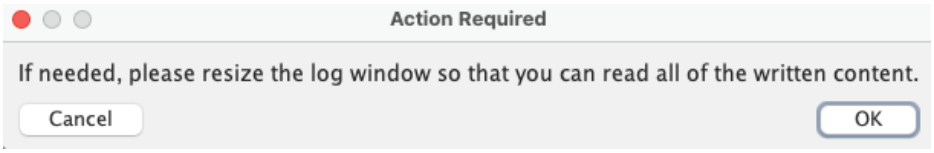
An ImageJ Macro for Analyzing 96 Well Hemagglutination Plates
https://www.youtube.com/watch?v=EF8ssYyJGDY

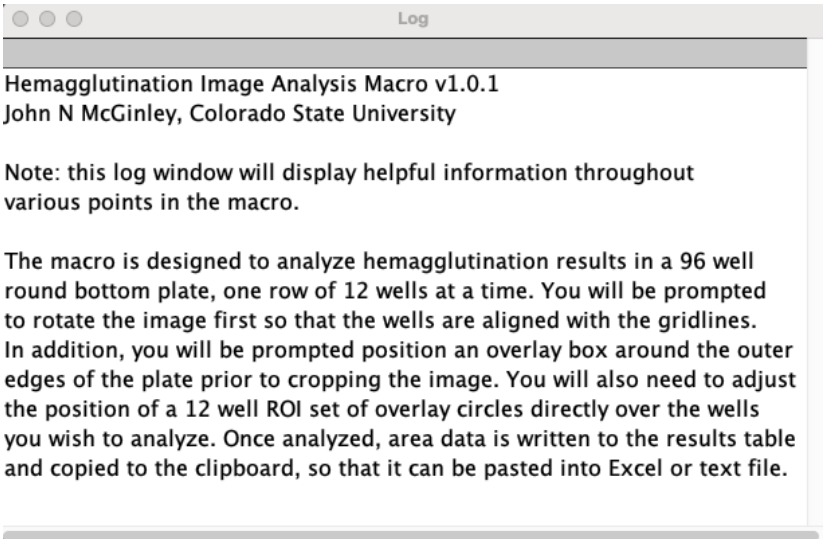
The macro and associated files can be downloaded from GitHub,
https://github.com/mcginleyj/hemagglutination

If ImageJ is not already installed go to https://imagej.net/ij/download.html. Download and install the appropriate version of ImageJ for your operating system.

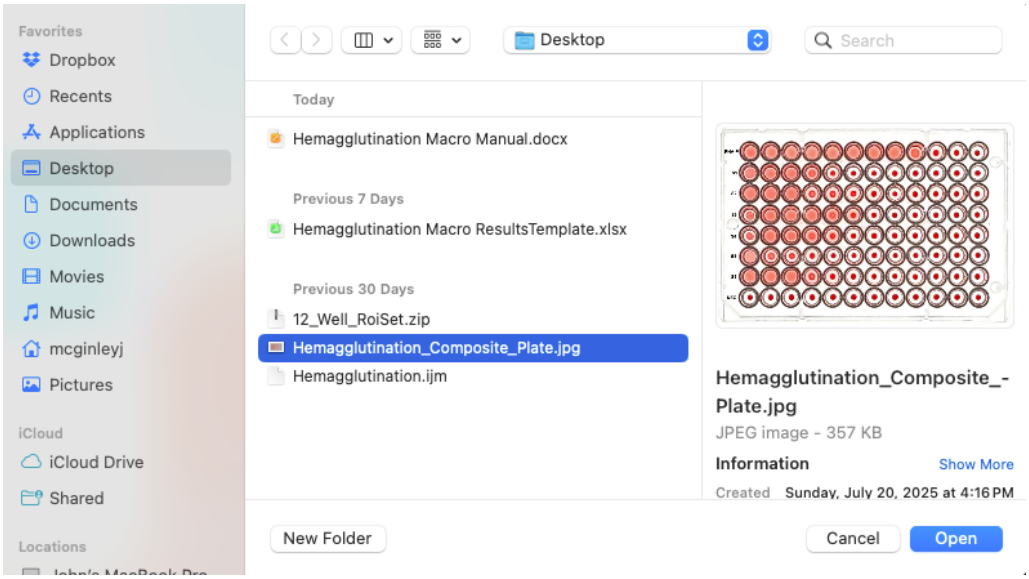
To run the macro, open ImageJ and using the menu, choose **Plugins, Macros, Run...** Browse to the **hemagglutination.ijm** file on your computer and click Open.
Note: the macro, associated files and plate images should be downloaded and saved on the local computer and not accessed using a network file share.

When running the macro you will first be prompted to resize the log window as useful information will be displayed in the window throughout various points during execution of the macro.





After resizing the log window and clicking OK, you will then be presented with a file browsing window with a prompt to open a hemagglutination plate image file.

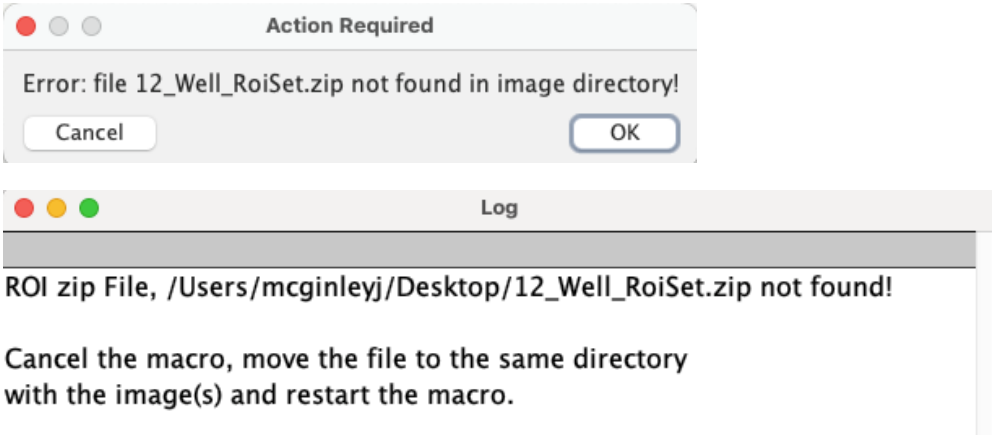


Note: this macro is designed to work with images in JPEG (.JPG) file format. The image file Hemagglutination_Composite_Plate.jpg is a composite image file for demonstration purposes that was created using images of different hemagglutination plates from the same run. PHP-P is the positive control lectin in row A at the top. Samples S1-S6 appear in rows B-G, respectively and Neg is the negative control (PBS) in the bottom row (H) of the plate. You should always include a positive control and negative control with each assay

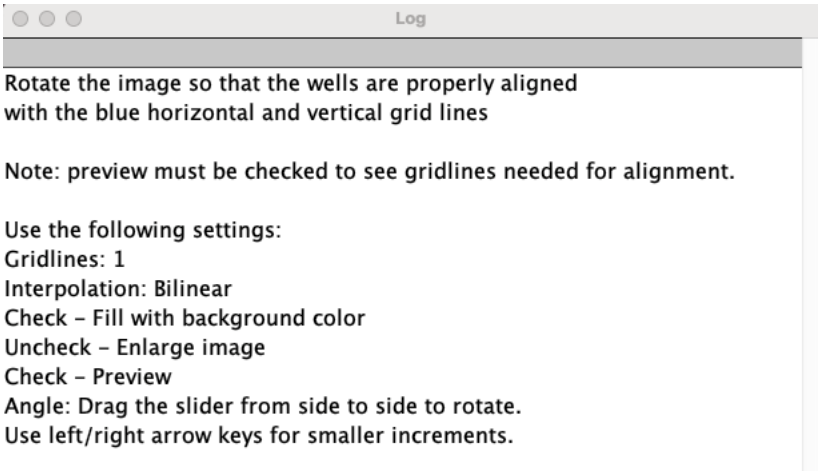
that is run. While images can be collected as soon as 2 hours after defibrinated rabbit red blood cells have been added to the plate, the clearest images are obtained after the plates have incubated overnight at room temperature.

******* Important *******

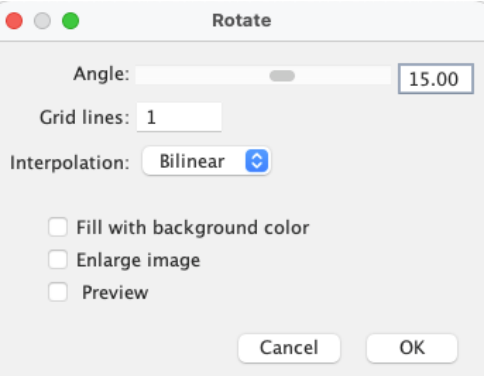
The zip file labeled "**12_Well_RoiSet.zip**" is required for the macro to function correctly and must be located in the same folder with the hemagglutination plate image(s) as shown in the image above. However, if it is not in the same folder with the image(s) you will be presented with an error prompt along with a message in the log window indicating that the macro should be canceled and the zip file moved to the same folder with the image(s) prior to restarting the macro as shown below.



Once the image file has been opened, the macro will prompt you to rotate the image so that well rows and columns are aligned with the blue horizontal and vertical crosshair gridline overlay on the image. Settings for rotation are displayed in the log window below.

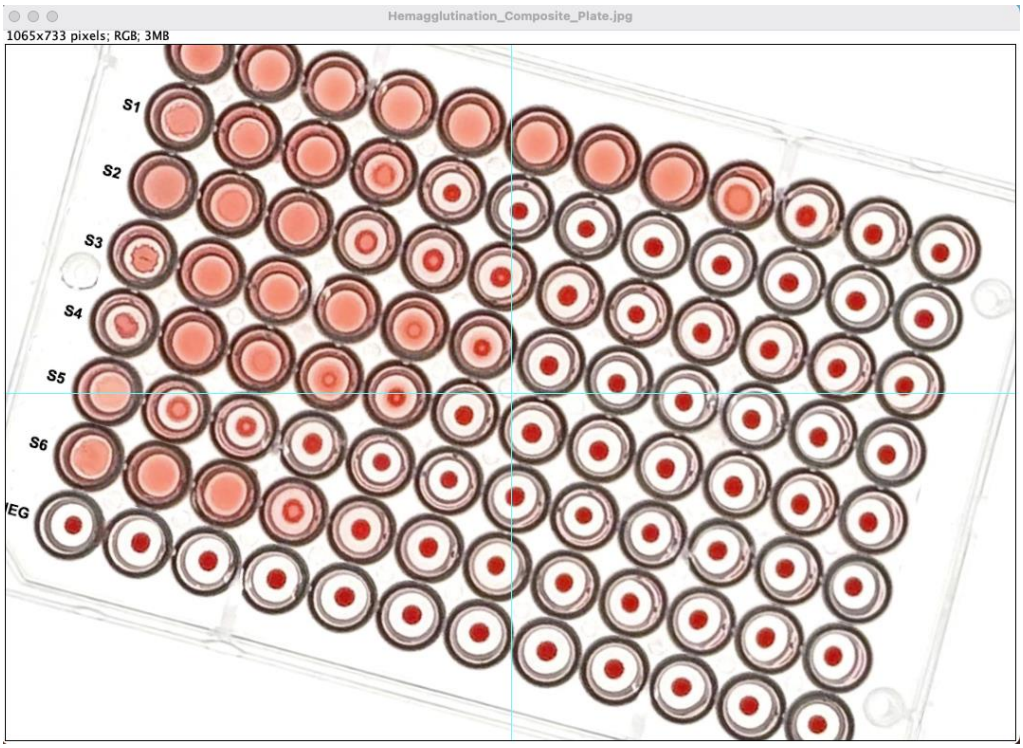


When starting a new session of ImageJ the angle in the rotate settings window will default to 15.00. The fill background, enlarge image and preview check boxes are unchecked by default.



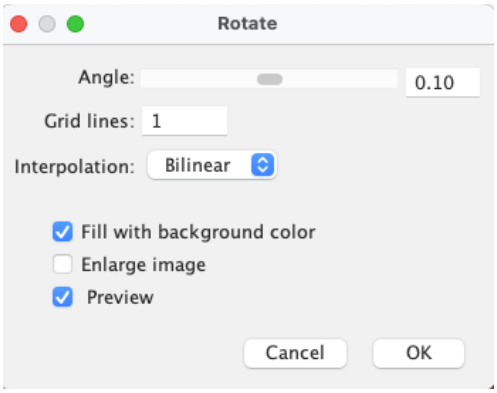
Note: the "Fill with background color" and "Preview" check boxes must be checked to see the rotation settings and grid overlay in the plate image.

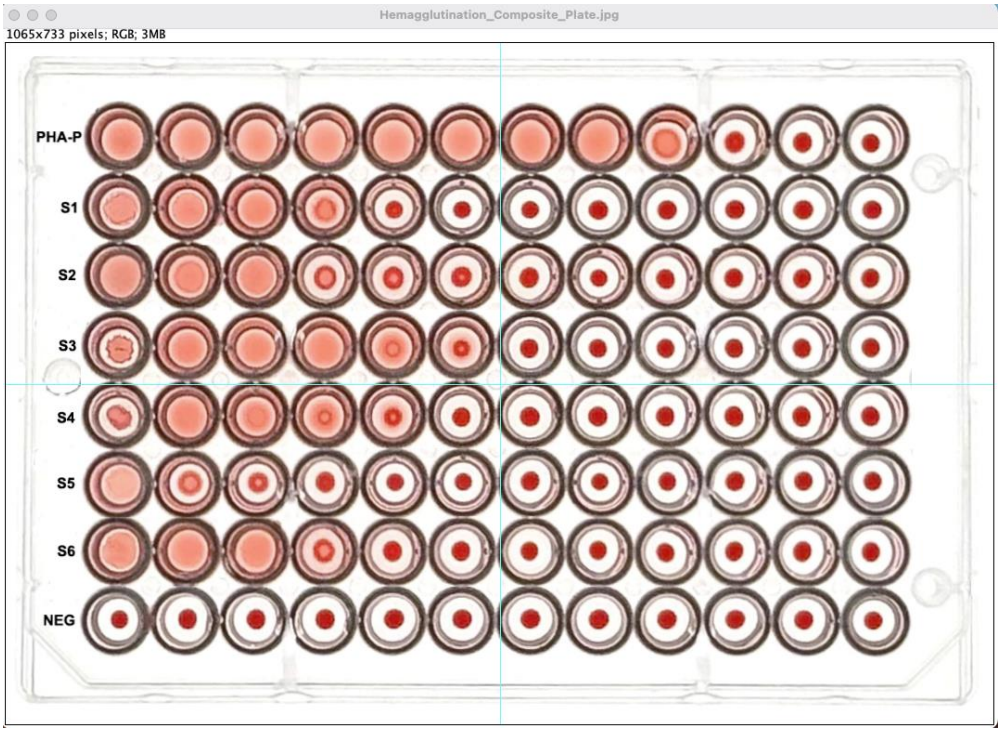
Below is an example of a plate image with the default angle rotation settings of 15.00 (both "Fill with background color" and "Preview" check boxes are checked, gridlines: 1, interpolation: bilinear and enlarge image is unchecked).



Note: The macro temporarily changes the ImageJ color settings for the background from the default of black to white and the selection color from the default of yellow to cyan. These settings will revert back to the default settings toward the end of the macro.

Drag the Angle slider to the left or right to change rotation. Use the left and right arrows on the keyboard to make smaller incremental changes in the rotation angle. When the row and column of wells in the plate image adjacent to cyan colored crosshairs are correctly aligned horizontally and vertically as show below, click OK to accept the changes.





Once the image has been rotated a cyan rectangle overlay will appear in the center of the plate image and you will be prompted to drag the corners of the cyan rectangle to the outer edges of the plate.

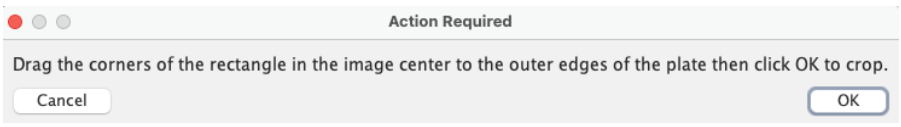


Plate image with cyan rectangle in the center of the image.

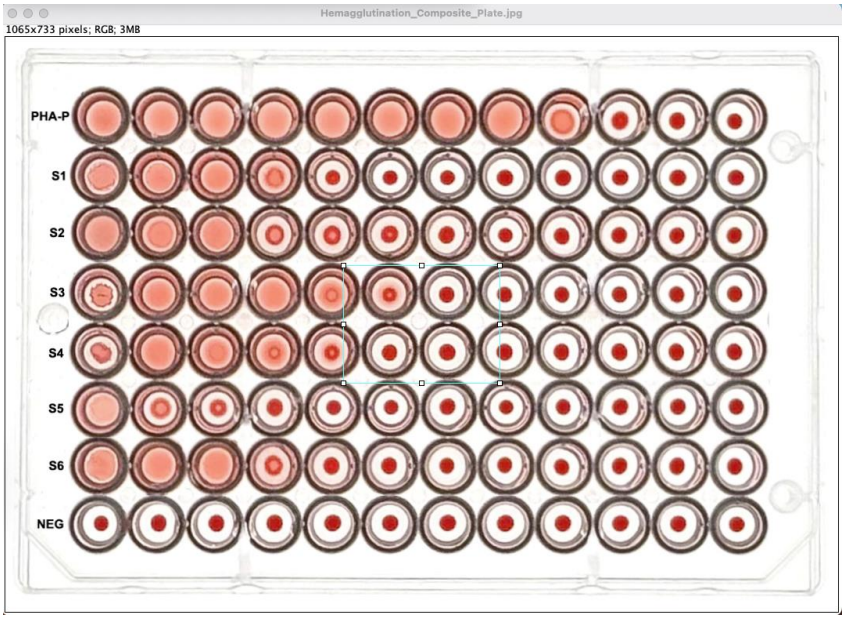
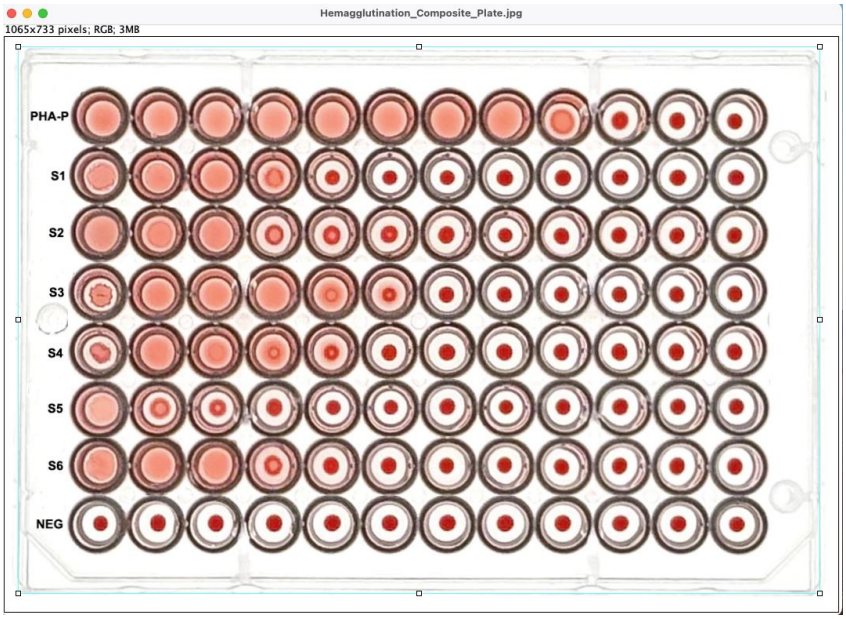
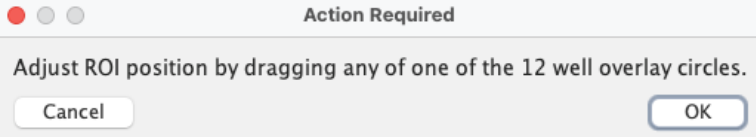


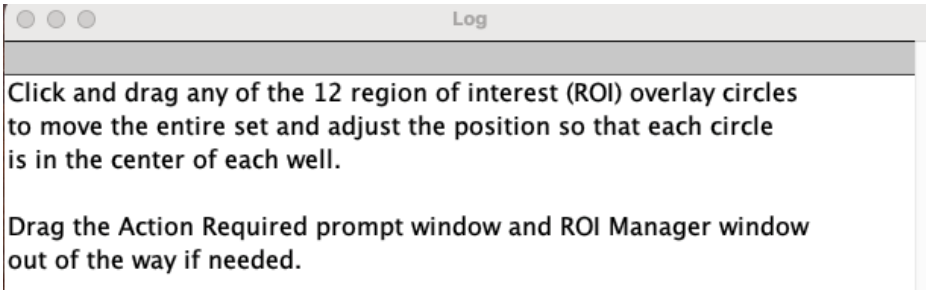
Plate image with the cyan rectangle overlay resized to include the outer edges of the plate.

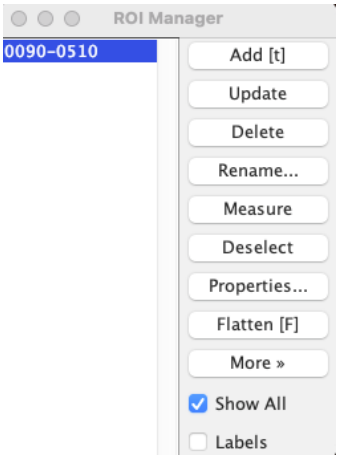


Once the cyan rectangle has been resized to outer edges of the plate click OK in the prompt, which will crop the image to the area occupied by the cyan rectangle. *Note: the rotation and cropping changes will be saved to a new temporary file in the image folder and not affect the original plate image.*

Next, the macro will convert the temporary image file from color to 8-bit grayscale. The Region of Interest (ROI) Manager window will be displayed and the file "12_Well_RoiSet.zip" containing 12 ROIs (one for each well in a single row of a 96 well plate) will be loaded into the ROI manger and displayed on the image.



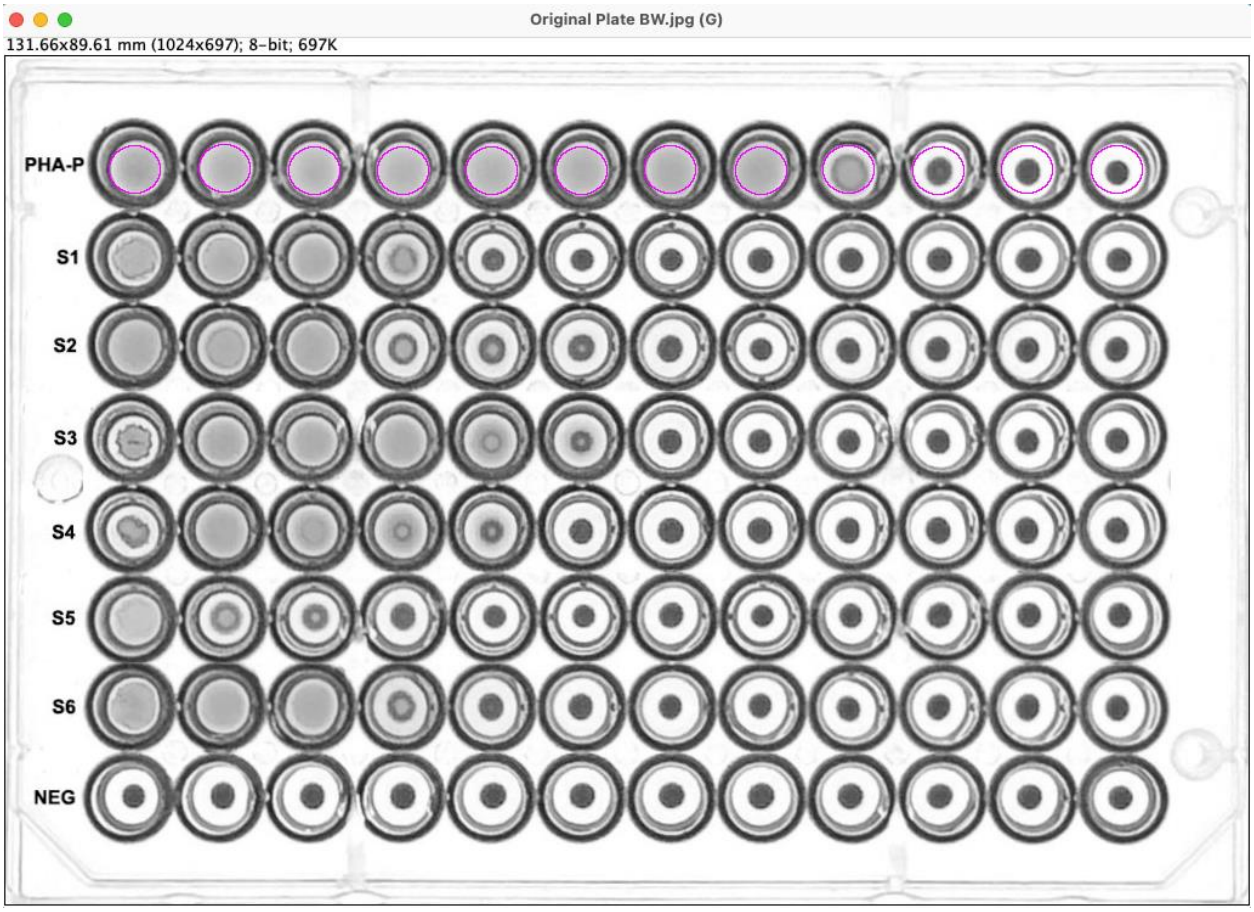




The macro combines the 12 separate ROIs into a single ROI. The entire set of 12 ROIs can then be moved in unison by clicking in the center of one of the ROIs and dragging the ROI set as needed. Position the ROI set directly over the well bottoms in the top row of the 96 well plate as shown below. The ROIs do not need to cover the entire well bottom.

******* IMPORTANT *******

The file **12_Well_ROIset.zip** provided with this macro was made specifically for use with the captured example plate images that go with this manual. **You will need to create your own 12 well ROI set using plate images that you have captured.** This can easily be done and a step-by-step procedure is listed in appendix A – How to Create a 12 well Region of Interest (ROI) Set. In addition, **you will also need to set the scale for your plate images**, and the procedure for that can be found in appendix B – Setting the scale for hemagglutination well measurements.



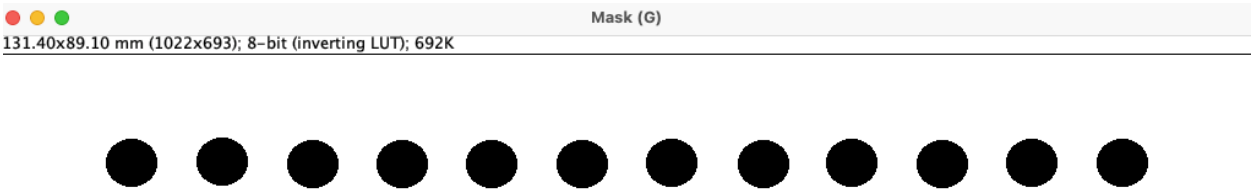
Once the ROI set (purple colored circles) has been positioned over wells 1-12 in the top row (A) of the plate image, click OK.

Note: if you are having trouble moving the ROI set and they appear as cyan colored circles as shown below, try double clicking inside one of the ROIs to turn the color outline from cyan to purple, which should allow you to move the entire ROI set in unison. If that doesn't work make sure the ROI number in the ROI manger is selected, which should highlight the number in blue and turn the ROI set from cyan to purple.

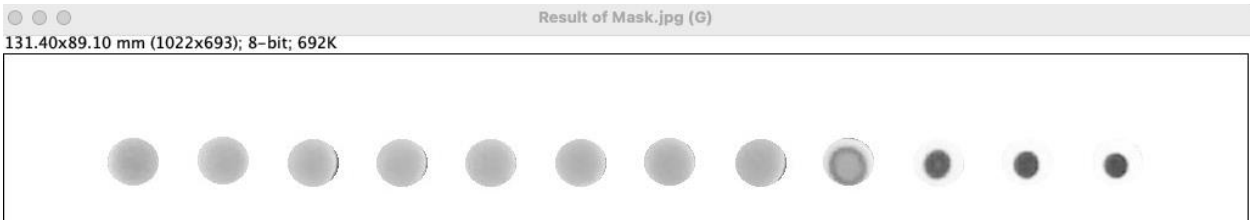
Deselected 12 well ROI set (cyan colored circles) – ROI position cannot move.

Selected 12 well ROI set (purple colored circles) – ROI position can move.

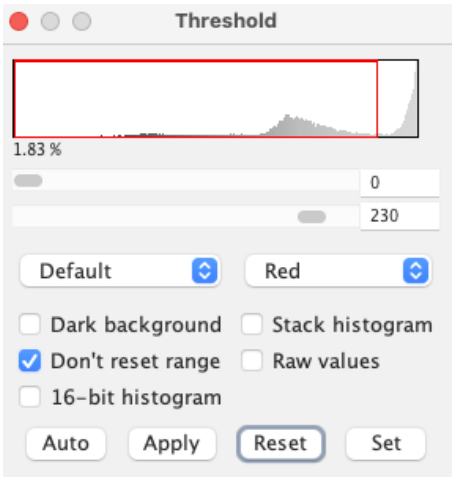
The macro will create a binary mask based on the 12 ROIs as shown below.



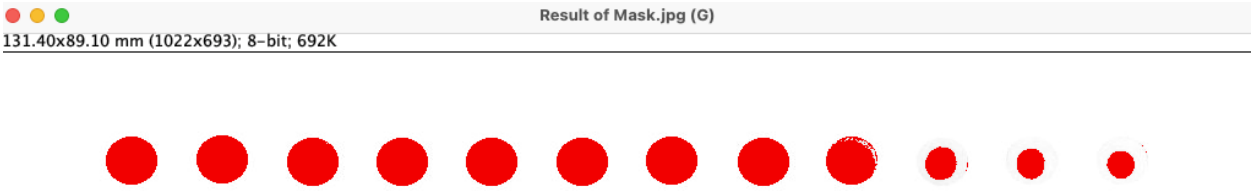
The binary mask is used to create a new image mask that retains only the 12 well bottoms from the 8-bit grayscale image on a white background as shown below.



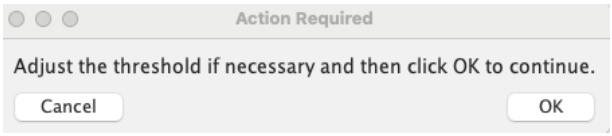
A threshold of 0-230 based on a grayscale range of 0-255, where 0 is black and 255 is white, is set.



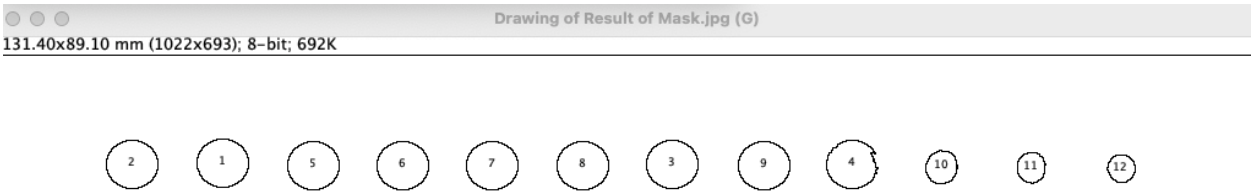
Threshold of 0-230 (red overly) applied to the mask image.



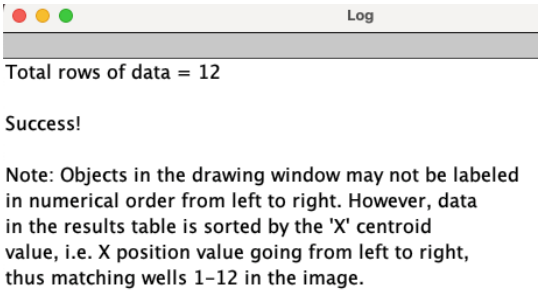
You will also have the opportunity to manually adjust the threshold, if needed.



The macro uses the Analyze Particles function in ImageJ to evaluate the 12 well bottoms based on the threshold setting above. The analyzed particles are displayed as circumscribed drawings of each well as shown below. *Note: the 12 number labeled particles in the drawing may or may not appear in numerical order from the left to right. However, in addition to the area measurement the centroid measurement (X & Y position of each particle or well in this case) is also measured. The data displayed in the Results table is sorted based on the X centroid measurement ensuring that area data in the Results table is correctly aligned with wells 1-12 (left to right) in the plate image.*



If 12 rows of data are listed in the Results table a “Success!” message is displayed in the log window as shown below.

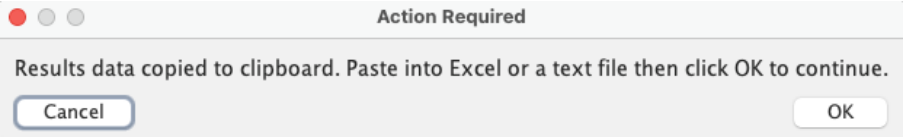


Results table showing area data sorted by X centroid (lowest to highest).

Results

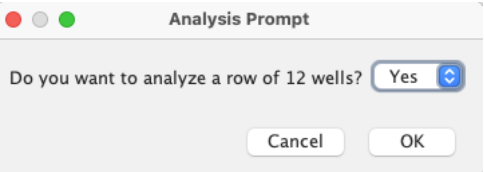
	Area	X	Y
1	22.041	13.847	11.560
2	22.058	23.512	11.427
3	22.058	33.174	11.691
4	22.075	42.710	11.690
5	22.075	52.245	11.690
6	22.075	61.910	11.690
7	22.075	71.445	11.559
8	22.075	81.241	11.690
9	21.034	90.573	11.604
10	9.246	100.303	11.998
11	7.609	109.870	12.022
12	6.636	119.485	12.119

Data from the Results table is automatically copied to the clipboard and can be pasted into Excel or a text file.

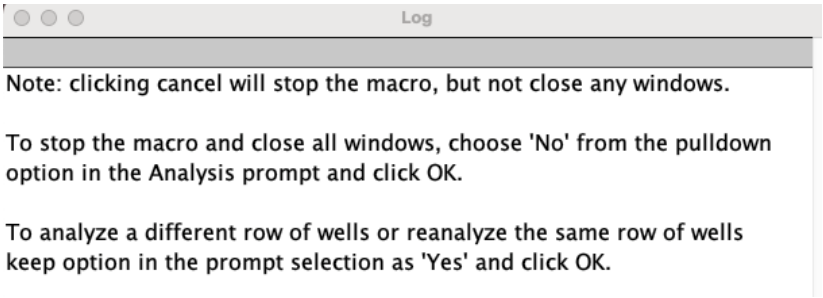


An Excel file labeled **Hemagglutination_Macro_Results_Template.xlsx** is provided for both data collection and summary. Instructions on how to use the file can be found in Appendix C - Data Collection and Summary Excel File Template.

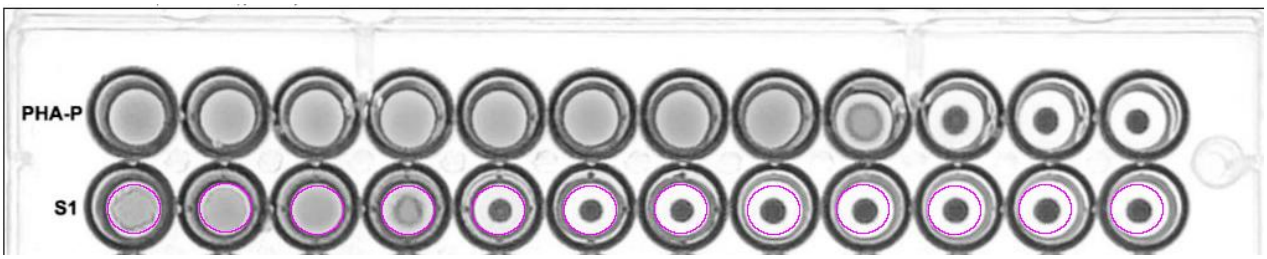
Once the OK button has been clicked you will be presented with a new prompt asking if you want to analyze a row of 12 wells with the answer defaulted to “Yes”.



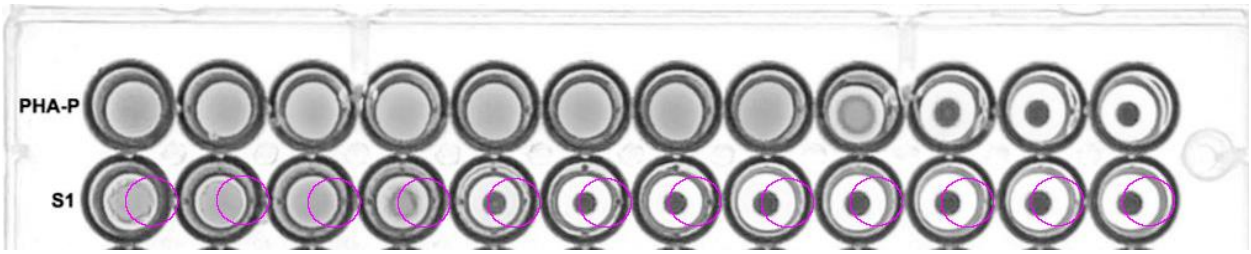
The log window displays a message indicating what will happen if you cancel the macro at this point. If you do not wish to analyze a different row of wells or re-analyze the same row of wells, select “No” from the pulldown option list and click OK, which will clear all windows and revert changed settings.



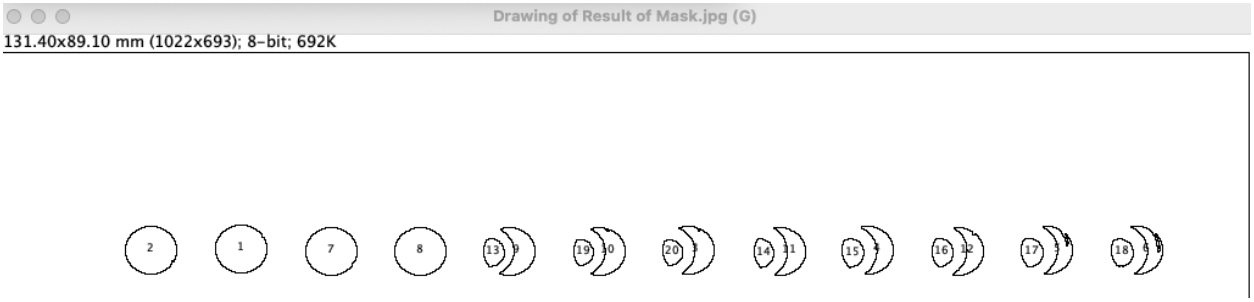
If “Yes” is chosen at the prompt and OK is clicked, you will be prompted to reposition the ROI set in the image, which can be moved to any row of wells in the plate. In the following example, the ROI set has been moved to the second row of 12 wells (S1). Once positioned, click OK and the analysis portion of the macro is executed as listed above.



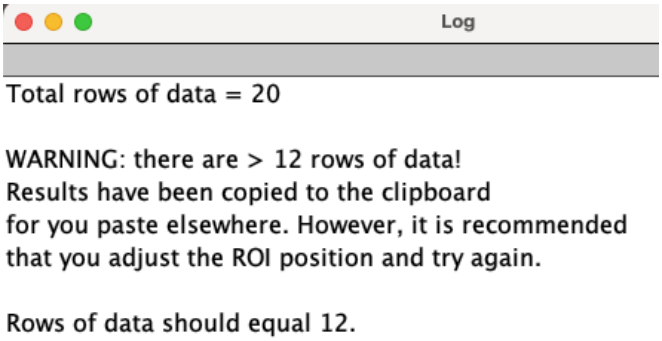
There may be instances where positioning of the 12 well ROI set may be offset by mistake. Below is an extreme example showing the 12 well ROIs offset to the right.



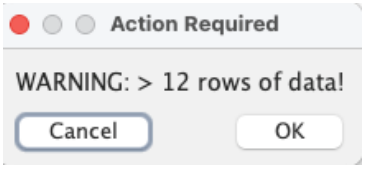
When the analysis is run more than 12 particles are detected as shown in the drawing image below.



The log window displays the total rows of data and a warning message.



You are also prompted with a warning prompt indicating the number of rows of data is > 12.



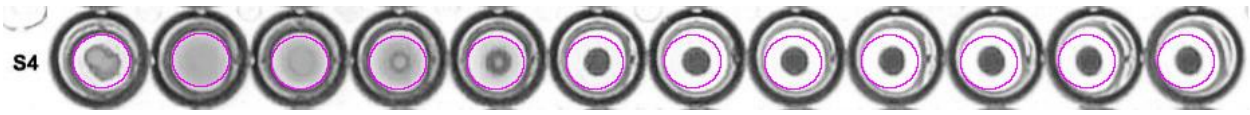
Once you click OK you will be presented with the analysis prompt asking if you want to analyze a row of 12 wells. You can then attempt to reposition the 12 well ROI set on the same row and try to analyze again. If it is still finding more than 12 particles (wells) in the analysis, try again, shifting the ROI set a little to left or right, up or down. If it catches a

small portion of an edge of a well bottom that should automatically be discarded during analysis. However, if too much of the edge is included as in the example above it will be retained during analysis.

There may also be instances where the analyze particles function will automatically toss out one or more wells resulting in < 12 rows of data. Sample rows S3 and S4 included in the composite plate image are examples of this where the default threshold setting of 230 shows numerous tiny red pixels between the periphery and the center portion of the first well. The analyze particles function is set to exclude edges and depending on how the ROI set is positioned with these particular sample rows it may or may not result in 12 well measurements.

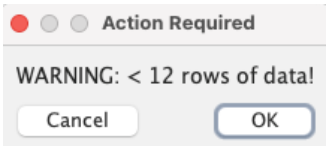
You will notice in the example below of sample S4 that wells 6-12 have partial semicircular shape of red pixels on the left representing the left edge of those wells. Likewise, the first well also has a semicircular shape of red pixels at the periphery, but unlike wells 6-12 there are also some red pixels that lie between the edge and center portion of the well. When the analyze particles function runs it discards the semicircle edge portions in wells 6-12, leaving the center portion of each well intact. However, when it tries to apply the same rule to the first well it ends up discarding the entire well.

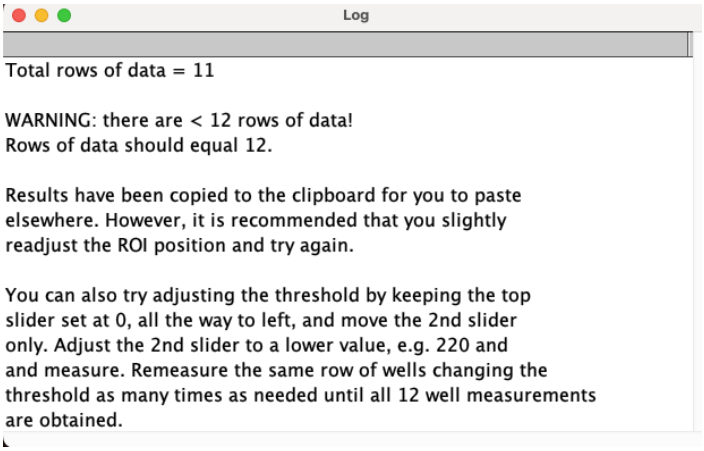
Sample row S4 grayscale image with 12 well ROI set overlay.



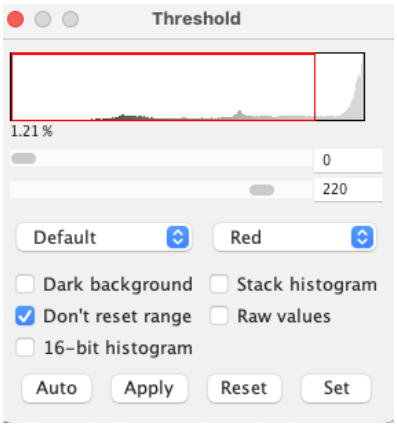
Sample row S4 with threshold set a 0-230 (default).

Analyze particles drawing shows the first well has been discarded resulting in < 12 rows of data along with a warning prompt and message in the log window.





You can try slightly repositioning the ROI set, focusing on the troublesome well and see if that solves the problem. If it does not, try reducing the threshold value 5-10 points and see if that helps. In the example below the threshold for sample S4 has been reduced from 230 down to 220.

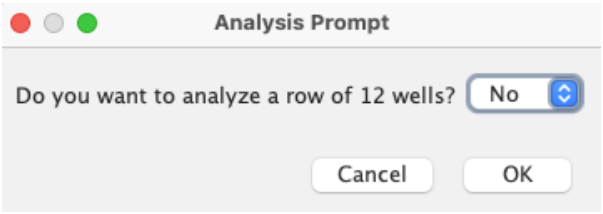


You will notice that the threshold area in the first well has fewer red pixels between the center of the well and the periphery. When the analyze particles function is run this time the edge of the first well is discarded leaving the center portion intact, so that all 12 wells are represented in the drawing below. Notice that the reduced threshold value has eroded a small portion of well 5 and the outer edge is discarded in the drawing outline, so reducing the threshold can be helpful, but you don't want to go too low. If reducing the threshold is not working then try increasing it slightly above 230, but not too much. *Note: raising the threshold all the way to 255 (white) will threshold the entire area of the image window resulting in no area data measurement.*

Sample S4 threshold set at 0-220.

Sample S4 analyze particles drawing (threshold set at 0-220) showing 12 wells.

Once all rows of the plate image have been analyzed you can choose "No" in the analysis prompt, which will close all open images and windows, delete temporary image files and revert color changes.



Note: The default measurement parameters in the macro are area and centroid, but this can be modified to include other measurements as listed in Appendix D - Changing Measurement Options.

Capturing good quality plate images is essential for the macro to work correctly and in the end it all comes down to garbage in, garbage out. Images should be captured using a lightbox with a white opaque diffusing panel between the light source and the bottom of the hemagglutination plate and the overhead room lights turned off and window blinds closed to avoid glare. In addition, the plate lid should be removed when capturing images. Do not capture images where the plate has been laid on top of a sheet of white paper with lamp lighting from above or angled from the sides as the quality will not be good. Illuminating the plate from below as is done with a lightbox, will provide quality images with sharper detail of the plate wells.

A suggested LED lightbox and tripod with an elongated smartphone camera mount arm are presented, which can be purchased online at reasonable cost. These along with a good smartphone camera are all that is necessary for acquiring high quality plate images. In addition, those wanting to build their own LED lightbox can also find suggested parts. All of these materials can be found in Appendix – E Lightbox and Image Capture.

Appendix A

How to Create a 12 well Region of Interest (ROI) Set

The file **12_well_RoiSet.zip** that comes with the hemagglutination.ijm ImageJ macro was created using images of round bottom hemagglutination plates (cat. 12565214, Fisher Scientific, Waltham, MA, USA) using an iPhone 16 camera with 2X zoom, mounted on a tripod with a fixed camera height of 75 cm above the lightbox.

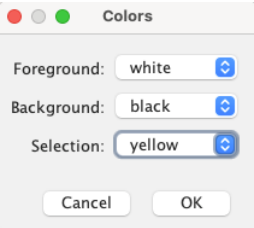
***** **Important** *****
Conditions will likely be different for each user, e.g. plate manufacturer, camera type, camera height, etc. Therefore, each user must create their own RoiSet file using their images. Otherwise, the macro will not work as intended, i.e. the well overlays in the RoiSet will not match the size and position of a 12 well row in the 96 well plate.

Video: Creating a 12 Well ROI Set for Analyzing Hemagglutination Plates in ImageJ
https://www.youtube.com/watch?v=d4BtTiAVIGo

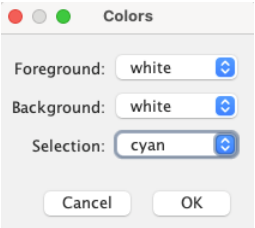
Open the ImageJ app.

Change colors (Edit, Options, Colors...). Default colors (Foreground: white, Background: black, Selection: yellow). Change background to "**white**" and selection to "**cyan**" then click OK. *Note: these colors can be changed back to the default settings after the ROI set has been created.*

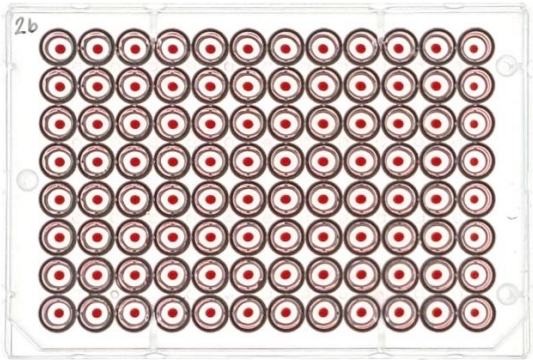
Default colors



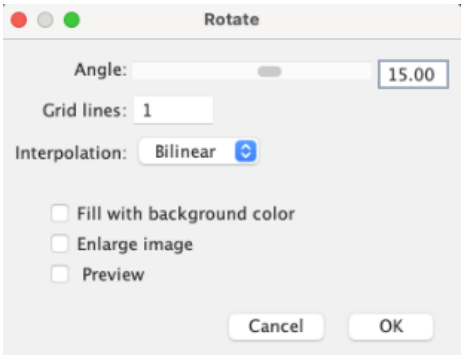
Changed colors



Open a 96 well hemagglutination plate image.

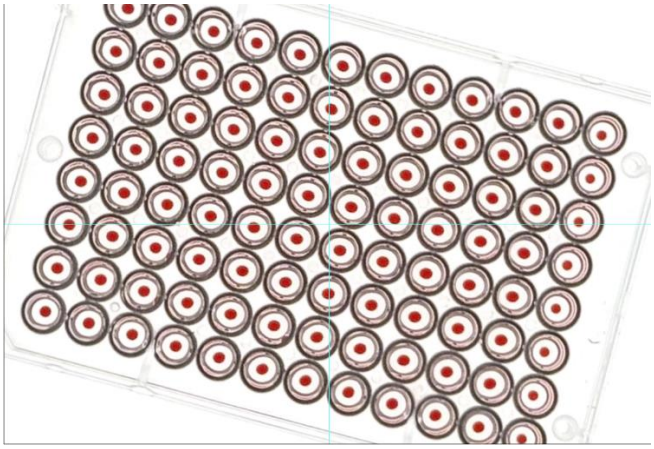


Rotate the image (**Image, Transform, Rotate…**).

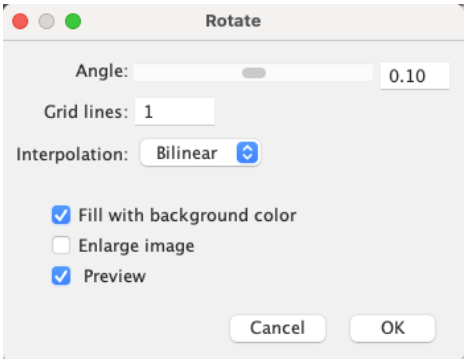


Use the following settings:
Gridlines: **1**
Interpolation: **Bilinear**
Check - Fill with background color
Uncheck – Enlarge image
Check – Preview

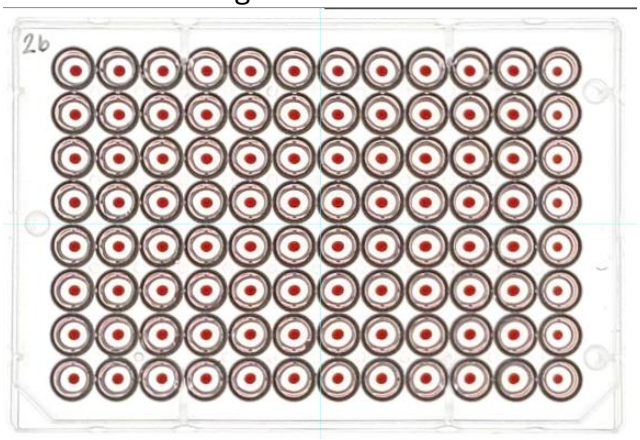
Note: preview must be checked to see the horizontal and vertical gridlines on the image and changes to the angle.
The angle will default to 15.00 and the image will appear tilted as in the example below (preview checked). If the background appears black, go back and set the background in colors to white.



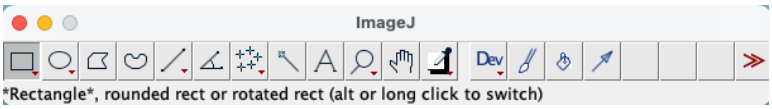
Drag the angle slider to the left or right to adjust the rotation angle of the image. Use the left and right arrows on the keyboard to adjust in smaller increments. Once image has been rotated where all of wells next to the horizontal and vertical gridlines are aligned, click OK.



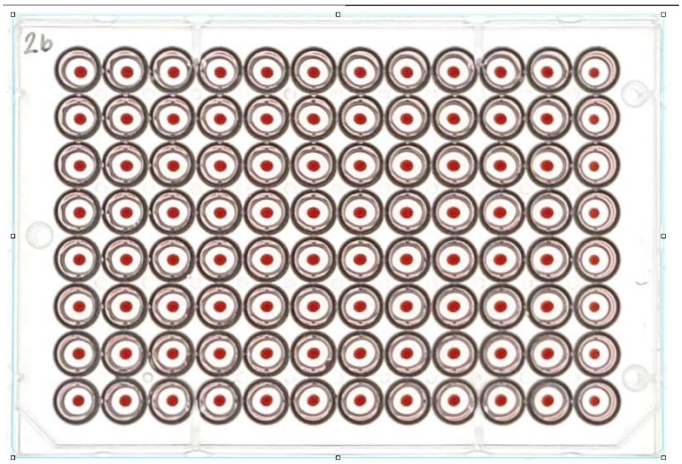
Final rotated image.



Using the **rectangle tool** from the toolbar...

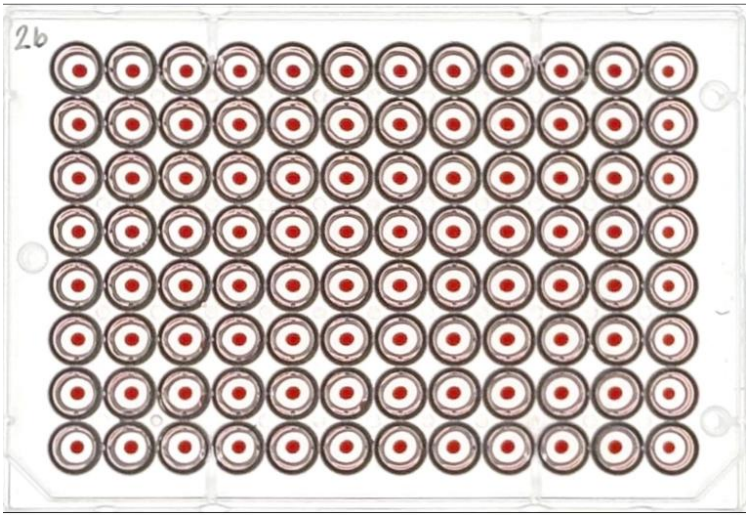


Draw a box around the outer edges of the plate.



Crop the image (**Image, Crop**).

Final cropped image.

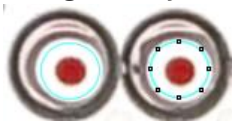


Using the **oval tool** from the toolbar…

Draw an oval around the bottom of the well in the top left corner of the plate.
Note: it does not need to cover the entire bottom of the well.

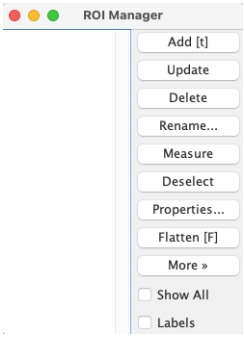
Add the oval to the overlay selection (**Image, Overlay, Add Selection…**).

Drag the overlay to the adjacent well to the right and add that well to the image overlay using the keyboard shortcut **CMD-B (Mac) or CTRL-B (Win)**.

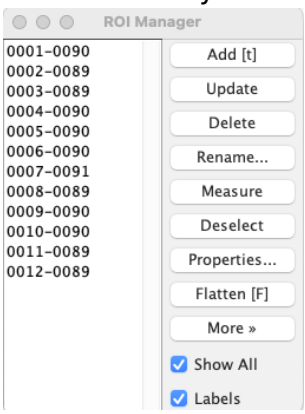


Repeat this process until all 12 wells in the same row have been added to the overlay then click elsewhere in the image to deselect the oval.

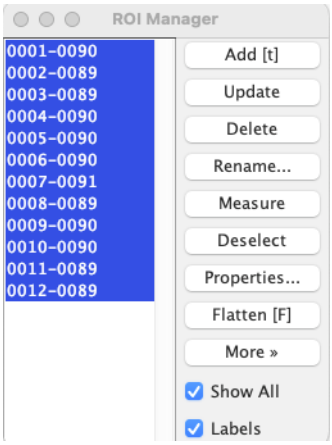
Open the ROI manager (**Analyze, Tools, ROI Manager...**)



Add the overlay to the ROI Manager (**Image, Overlay, To ROI Manager**).

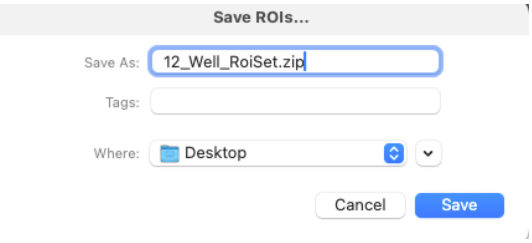


In the ROI Manger Window, click the top ROI, shift key down and click the bottom ROI so that all ROIs in the list are highlighted blue.



In the ROI Manager window, click **More>> Save...**

Save the file to the desktop using the file name "**12_Well_RoiSet.zip**".



To ensure that the ROI set was saved correctly. Make sure all 12 ROIs are still selected in the ROI Manger (highlighted blue) and click delete to remove all of the ROIs. Add the 12 ROIs back to the image by choosing **More>>Open...** Select the **12_Well_RoiSet.zip** file from the desktop that you just created and click Open. In the ROI manager make sure that the Show All option is checked. The 12 well ROIs should be visible and aligned with the 12 well bottoms in the first row of the plate.

Appendix B

Setting the Scale for Hemagglutination Well Measurements

To ensure that area measurements are accurate each user will need to measure the diameter of the well bottom in a plate image that they have collected. It is imperative that plate images be captured using the following conditions:

Round Bottom Plate: use a round bottom plate of your choice, but be consistent, using the same manufacturer and catalog number of plate.

Camera: use the same camera and zoom settings each time whether it is a smartphone camera or other digital camera.

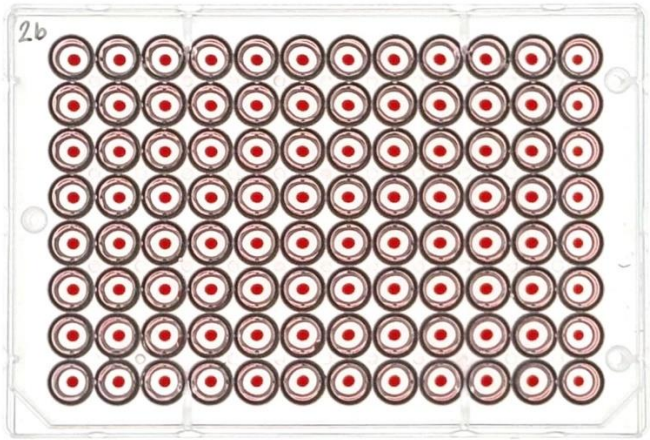
Camera height: use the same fixed camera height each time. A tripod with a camera mount placed directly over the plate on the lightbox is highly recommended. A camera height of 75 cm above the light box will minimize parallax, but will not completely eliminate it.

Lighting: use the same lighting conditions each time, e.g. lightbox with overhead room lights turned off.

If these conditions are met then setting the scale measurement should only need to be done once. However, if any of the above conditions change you should go though the procedure again to obtain updated values.

The following is also covered toward the end of the following video (14:56):
Creating a 12 Well ROI Set for Analyzing Hemagglutination Plates in ImageJ
https://www.youtube.com/watch?v=d4BtTiAVIGo&t=896s

Start ImageJ and open a hemagglutination plate image that you have captured.

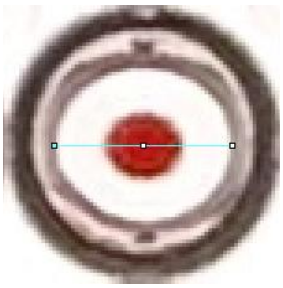


Using the **magnifying glass** tool from the toolbar...

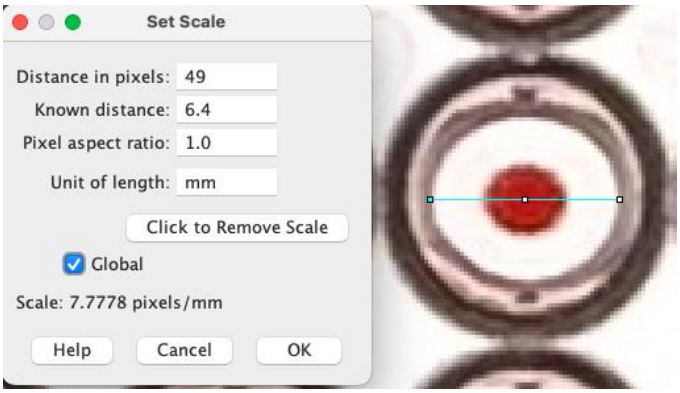
Click in the center of the image to increase the zoom from 100% to 300%.

Using the **straight line tool** from the toolbar...

Choose a well from the center of the plate and draw a horizontal line from the left edge of one well bottom to the right edge as shown in the example well below.



Set the scale (**Analyze, Set Scale...**).



Distance in pixels: this is the value in pixels of the horizontal line that was drawn in the image using the straight line tool.
Known distance: this values will need to be entered and can be found in the round bottom plate spec sheet from the manufacturer.
Pixel aspect ratio: should be kept at 1.0.
Unit of length: should be set to mm.
If taking measurements manually using more than one plate image per session, check the box marked global.

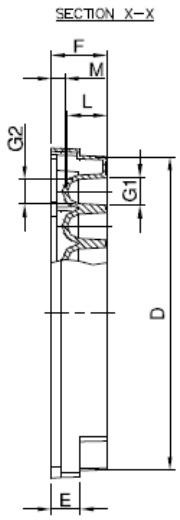
Two values will need to be changed in the **hemagglutination.ijm** macro file. Open the file using **TextEdit (Mac) or Notepad (Win)**.

```
// ********** Configuration Start **********
WellDist = 49;       // Diameter of well bottom measured in pixels. Must be determined by user as camera height, resolution and type of plate will vary.
KnownDist = 6.4;     // Known diameter of the well bottom in mm according to plate manufacturer spec sheet.
```

At the top of the macro are several lines listed under the section marked ********** **Configuration Start** **********. The variable **WellDist** is the distance in pixels from the Set scale window. **KnownDist** is the known diameter of the well bottom in mm according to the plate manufacturer. **Update both WellDist & KnownDist values and save** the macro prior to measuring your plates. As mentioned above, if all image capture conditions are kept consistent this should only need to be done once. However, if conditions change the process should be repeated to ensure measurements are correct.

The plates that our laboratory uses are Thermo Scientific™ Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates (cat. 12565214, Fisher Scientific, Waltham, MA, USA). The spec sheet for these plates list the well bottom diameter (G2) as 6.4 mm and this is the value entered for the **KnownDist** variable. Plates from other manufacturers will be slightly different, so find the spec sheet for your plates and change the value in the macro accordingly. Below are screenshots of the spec sheet.

PLATE



	MM
A, BASE LENGTH	127,7
B, BASE WIDTH	85,5
C, TOP LENGTH	123,3
D, TOP WIDTH	81,2
E, FLANGE LONG SIDE	7,5
F, OVERALL HEIGHT	14,4
G1, DIA TOP	Ø7,1
G2, DIA BOTTOM	Ø6,4

Appendix C

Data Collection and Summary Excel File Template

An Excel file labeled **Hemagglutination_Macro_Results_Template.xlsx** is provided to accept pasted values from the ImageJ Results table. Data is pasted into the tab labeled **Raw_Data_12_Wells**. In the demo example the sample name appears in Column A followed by the well number, area measurement (mm), X and Y centroid values. Data is pasted into the sheet 12 rows at a time as shown in the example below.

	A	B	C	D	E
1	**Sample**	**Well**	**Area**	**X**	**Y**
2	PHA-P	1	22.058	13.715	11.558
3	PHA-P	2	22.075	23.38	11.429
4	PHA-P	3	22.092	33.047	11.69
5	PHA-P	4	22.075	42.58	11.69
6	PHA-P	5	22.075	52.114	11.69
7	PHA-P	6	22.092	61.778	11.69
8	PHA-P	7	22.075	71.314	11.559
9	PHA-P	8	22.075	81.11	11.69
10	PHA-P	9	21.034	90.443	11.624
11	PHA-P	10	9.28	100.186	11.853
12	PHA-P	11	7.609	109.738	11.891
13	PHA-P	12	6.756	119.357	11.992
14	S1	1	21.734	13.721	21.208
15	S1	2	22.058	23.379	21.095
16	S1	3	22.075	33.045	21.355

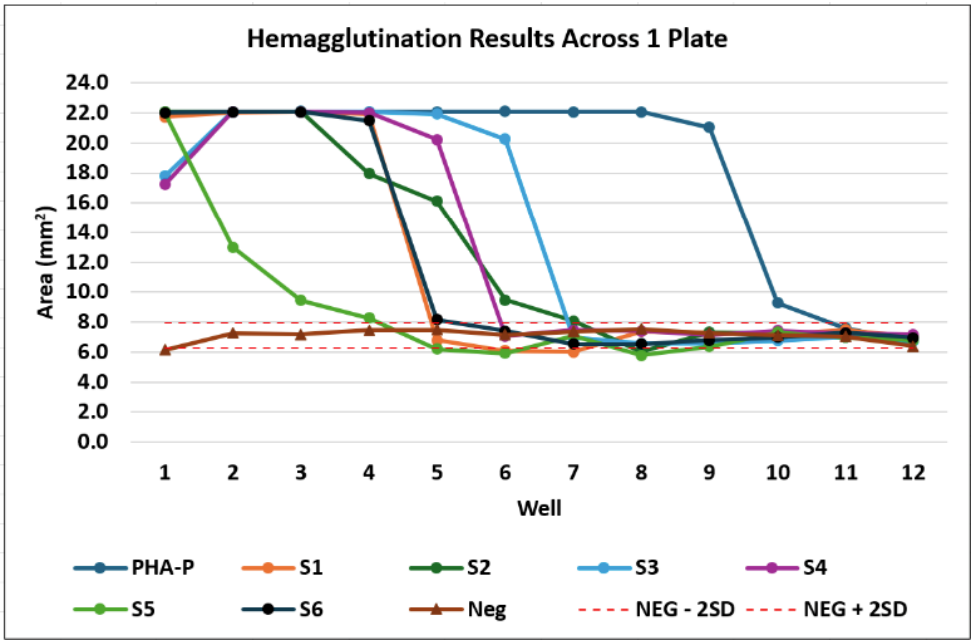
The second tab in the Excel file labeled **Summary_12_Wells** displays a table that pulls values from the raw data tab. The 12 Neg (negative control) sample wells are used to calculate mean Neg ± 2SD as shown in columns J and K below. Conditional formatting is automatically applied to the sample well values for the PHA-P positive control and the six samples S1-S6. Cells shaded red (columns B-H) indicate positive wells where the area measurement is > 2SD above the mean Neg value (listed in column K).

	A	B	C	D	E	F	G	H	I	J	K
1	**Well**	**PHA-P**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**Neg**	**NEG - 2SD**	**NEG + 2SD**
2	**1**	22.058	21.73	22.075	17.793	17.23	22.041	22.024	6.159	6.27	7.98
3	**2**	22.075	22.06	22.075	22.075	22.075	12.999	22.075	7.267	6.27	7.98
4	**3**	22.092	22.08	22.075	22.075	22.075	9.451	22.075	7.182	6.27	7.98
5	**4**	22.075	21.94	17.964	22.075	22.024	8.274	21.478	7.455	6.27	7.98
6	**5**	22.075	6.79	16.121	21.939	20.233	6.21	8.154	7.489	6.27	7.98
7	**6**	22.092	6.107	9.485	20.25	7.08	5.937	7.421	7.148	6.27	7.98
8	**7**	22.075	6.022	8.069	6.96	7.455	7.114	6.534	7.387	6.27	7.98
9	**8**	22.075	7.37	6.039	6.568	7.404	5.783	6.551	7.557	6.27	7.98
10	**9**	21.034	7.301	7.336	6.602	7.131	6.38	6.807	7.267	6.27	7.98
11	**10**	9.28	7.182	7.233	6.773	7.438	7.319	6.994	7.131	6.27	7.98
12	**11**	7.609	7.506	6.977	7.011	7.267	6.977	7.301	7.046	6.27	7.98
13	**12**	6.756	7.046	7.011	6.704	7.199	6.79	6.96	6.397	6.27	7.98

An HAU table is provided below the summary table. To find the HAU value for a given sample, find the last well number that shows a positive value (red shaded cell). In the example summary table above the last positive value for PHA-P is well 10 (area = 8.893). Look up the HAU value for well 10 in the PHA-P column of the HAU table below, which is 512 HAUs.

15	HAU Table							
16	**Well**	**PHA-P**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
17	**1**	1	1	1	1	1	1	1
18	**2**	2	2	2	2	2	2	2
19	**3**	4	4	4	4	4	4	4
20	**4**	8	8	8	8	8	8	8
21	**5**	16	16	16	16	16	16	16
22	**6**	32	32	32	32	32	32	32
23	**7**	64	64	64	64	64	64	64
24	**8**	128	128	128	128	128	128	128
25	**9**	256	256	256	256	256	256	256
26	**10**	512	512	512	512	512	512	512
27	**11**	1024	1024	1024	1024	1024	1024	1024
28	**12**	2048	2048	2048	2048	2048	2048	2048

Data from the summary table is also displayed as a graph as shown below.



Notice how samples S3 and S4 have a lower area measurement for well 1 compared to wells 2-4. This is a common issue with the hemagglutination assay and is reflective of artifact that is caused from the agglutinated red blood cells rolling up or folding back on themselves resulting in various smaller polygonal shapes instead a smooth, flat carpet of agglutinated red blood cells that cover the entire well. In some cases the area measurement may be just above the area of a negative well and may be seen in more than

one consecutive dilution well for a sample. If this is the case and subsequent dilution wells show an increase in agglutinated area as shown for samples S3 and S4 above, the beginning well values should be ignored as artifact.

A second set of tabs is provided in the Excel file labeled Raw_Data_24_wells and Summary_24_Wells. These tabs should be used when evaluating raw bean sample, which in several cases require samples to be serially diluted across two hemagglutination plates, i.e. serial dilutions beyond 12 wells. The process is similar to pasting data from 12 wells except that it is done using two plate images instead of one.

To make the data collection process easier, analyze all of the rows in the first plate image, pasting the first set of 12 well data at the beginning of each listed sample. When all samples for that plate have been analyzed open the second plate image containing serial dilution wells 13-24, and analyze all sample rows pasting the values in the 12 remaining blank spaces for each sample.

The sample wells in the raw data will have two sets of wells labeled 1-12 for each sample as the data has been collected from two different images. Once all the well rows from the second image have been analyzed, well data numbers from column G, repeating sets of 1-24, can be copied and the values pasted into column B to overwrite the two sets of repeating 1-12 well numbers for each sample, resulting in well numbers 1-24 for each sample.

Appendix D

Changing Measurement Options

Area is the most useful measurement for this type of assay. While min & max gray value, mean gray value and integrated density measurements are available in ImageJ they are not applicable as the assay is non-stochiometric and does not adhere to Beer-Lambert law. Nevertheless, if you would like to see these values they are already listed in the macro and can be enabled by editing the macro file using TextEdit (Mac) or Notepad (Win) as follows:

Default MeasureOptions (Area & Centroid) located at the end of the configuration section in the beginning of the macro.

```
MeasureOptions = "area centroid redirect=None decimal=3";    // Default well mea
// MeasureOptions = "area centroid mean min integrated redirect=None decimal=3";
// ********** Configuration End **********
```

To change MeasureOptions to include mean gray value, min & max gray value and integrated density, comment out the first MeasureOptions line by adding two forward slashes "**//**" at the beginning of the line then delete the two forward slashes from the beginning of the second line for MeasureOptions as shown below and save the file.

```
// MeasureOptions = "area centroid redirect=None decimal=3";    // Default well
MeasureOptions = "area centroid mean min integrated redirect=None decimal=3";  /
// ********** Configuration End **********
```

Below is example output from the Results table with the additional measurement options enabled.

Results

	Area	Mean	Min	Max	X	Y	IntDen	RawIntDen
1	22.075	180.657	155	229	13.714	11.298	3988.013	233770.000
2	22.075	186.696	167	230	23.380	11.167	4121.316	241584.000
3	22.075	185.429	61	227	33.045	11.429	4093.356	239945.000
4	22.075	186.320	105	230	42.580	11.429	4113.025	241098.000
5	22.075	186.252	157	227	52.114	11.429	4111.524	241010.000
6	22.075	184.052	134	224	61.780	11.429	4062.956	238163.000
7	22.075	181.675	130	221	71.314	11.298	4010.480	235087.000
8	22.075	178.485	95	221	81.110	11.429	3940.076	230960.000
9	21.017	171.148	64	230	90.436	11.344	3597.076	210854.000
10	9.212	131.691	77	230	100.050	11.721	1213.156	71113.000
11	7.660	120.165	71	230	109.615	11.764	920.431	53954.000
12	6.670	116.537	55	230	119.229	11.863	777.336	45566.000

Appendix E

Lightbox and Image Capture

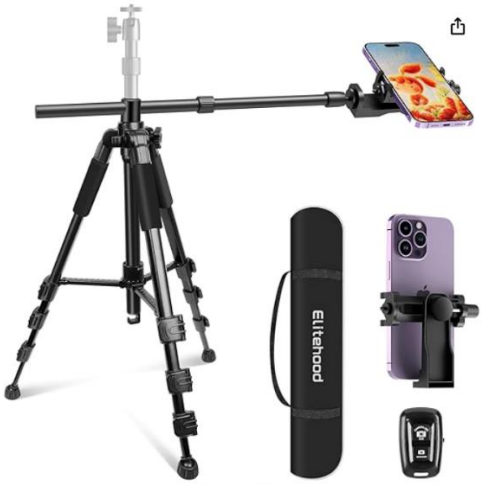
LED Lightbox

A suitable lightbox with a white diffusing panel should be used when capturing plate images, e.g. Gagne Porta-Trace 10” X 12” 2 LED light box, model 1012-2L or similar product should suffice. This a complete lightbox product and is available online.
https://porta-trace.com/product/stainless-steel-led-lightboxes/

Tripod

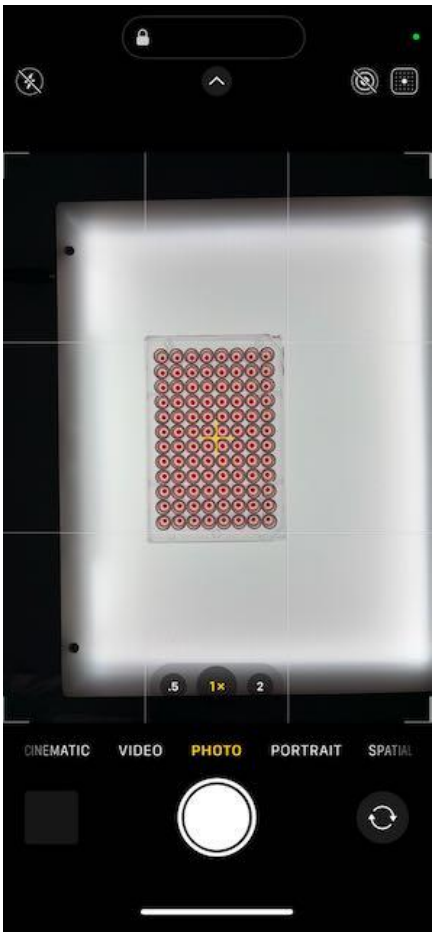
Elitehood Ultra-Stable Overhead Tripod for iPhone – Heavy Duty Aluminum iPhone Tripod Stand, 360°Adjustable & 25in Horizontal Long Phone Arm, Tall Phone Holder Stand Mount for Recording

https://www.amazon.com/dp/B0C2VBFG62



A smartphone is mounted on the tripod arm and placed over the lightbox. Capturing good quality plate images is essential for the macro to work correctly and in the end it all comes down to garbage in, garbage out. Images should be captured using a lightbox with a white opaque diffusing panel between the light source and the bottom of the hemagglutination plate and the overhead room lights turned off and window blinds closed to avoid glare. In addition, the plate lid should be removed when capturing images.

The camera height should be raised above the lightbox, e.g. 75 cm to minimize parallax in the captured images. Grid and level options should be turned on to ensure that the camera angle is level and the plate is square when capturing images. Once the image is in focus the Bluetooth remote that comes bundled with the tripod is used to acquire the image without having to touch the smartphone. Exposure of captured images should be increased to an appropriate bright level to visualize detail, e.g. 30 prior to exporting as JPEG files.



Note: zoom level is set to 1x in the smartphone camera app screenshot image above to show the entire lightbox, but actual images are captured using 2x zoom (camera height 75 cm above lightbox).

Optional DIY LED Lightbox

If you would rather make your own lightbox you can convert an old fluorescent porta-trace light box to LED as our lab did or you create a wooden frame with the same dimensions (10” X 12” X 5”). Note: functional fluorescent porta-trace lightboxes are not compatible with capturing images using a smartphone camera as the refresh rate of the fluorescent bulbs interferes with proper camera exposure. Below is a list of suggested supplies for making DIY lightboxes.

5V 10A power supply

BTF-LIGHTING AC100-240V to DC5V10A Max50W Suit 9A 8A 7A 6A 5A 4A 3A 2A 1A Device Transformer Power Adapter Converter with 5.5x2.5mm DC Output Jack for WS2812B WS2811 WS2813 SK6812 etc LED Strip/String.

https://www.amazon.com/dp/B01D8FM71S

LEDs

BTF-LIGHTING RGBW RGB+Natural White SK6812 (Similar WS2812B) 4 Colors in 1 LED Strip Individually Addressable 16.4ft 60Pixel/m 300LED Light Dream Color IP30 DC5V White PCB (No Adapter or Controller)

https://www.amazon.com/dp/B01N5ATQZT

Note: a total of 68 LEDs are arranged around the inside perimeter of the lightbox towards the top, pointing to the center of the box. If using a wooden frame, the interior of the frame should be painted white. In addition, a parabolic shaped piece of sheet metal painted white and placed in the bottom of the frame is preferred to help reflect LED light from the sides up through the white diffusing panel on top.

White diffusing panel

Rock Hard Plastics - 12" x 12" White Acrylic Sheet Lucite Plexiglass - (Actual Size 11.875" x 11.875" - .118" (1/8")

https://www.amazon.com/dp/B07MG8KTBX

The diffusing panel is placed on top of the frame allowing light from the LEDs to shine upward through the panel proving illumination to the bottom of the hemagglutination plates.

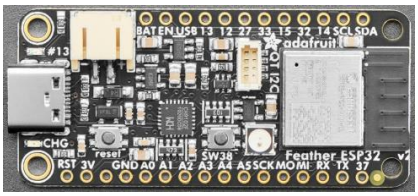
3 conductor wire (positive, ground, data)

80 Feet(24 Meter) 3 Pin LED Extension Wire 20 AWG, for WS2812B WS2811 Addressable Dream Color Tape Light Wiring,3 Conductor Electrical Cable for Digital IC Tape Light, Hook Up Wire

https://www.amazon.com/dp/B0829NPHF3

ESP32 Microcontroller

Adafruit ESP32 Feather V2 - 8MB Flash + 2 MB PSRAM - STEMMA QT
https://www.adafruit.com/product/5400



WLED software

https://install.wled.me/

The freely available WLED software can be flashed directly to the microcontroller via USB-C cable using the URL above with a compatible web browser like Google Chrome. This eliminates the need of writing code for the microcontroller. The software can be configured using a smartphone via the built-in Wi-Fi access point (WLED-AP). LED hardware and setup screens are shown below with number of type of LEDs along with the designated GPIO pin providing data from the microcontroller to drive the LEDs. Color can be saved as a preset that will load each time the microcontroller is booted.