Microscopy Pipeline Workflow - Image Acquisition

Purpose

This protocol summarizes the steps required to acquire experimental and control images for the Microscopy pipeline data generation for the Integrated Cell project.

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Required Equipment & Reagents

The following protocol has been optimized for the following equipment:

- Zeiss motorized AxioObserver for Spinning Disk (Part#4310079902, Carl Zeiss Microscopy, LLC)
- CSU-X1 scanhead, 6 position FW (Yokogawa, Co-innovation tomorrow)
- Laser Bench: 405nm, 50mW, 488nm, 50mW, 638nm, 75mW
- Emission Filter sets: BP 450/50, BP 525/50, BP 690/50
- Illumination carrier Tilt Back f/Observer Z1 for transmitted light.
- Incubator XL multi S1 with temp module S1, Co2 module S1, Heating Unit XL S2 (PECON)
- Camera: Hamamatsu Flash 4.0 V2 Plus
- Objectives: P-Apo 10x/0.45NA WD=2.0, C-Apo 100x/1.25NA W corr WD=0.25mm
- Software: Zen Blue 2.0
- 96-well plates prepared according to protocols listed below.

Related SOPs

The following SOPs are used in conjunction with this protocol:

- WTC culture v1.1
- Cell plating for imaging v1.0
- CellMask and NucBlue v1.0
- Fluorescent dye solutions for flat field correction
- Sub-resolution and focal check beads solution for alignment and PSF measurements

Preparation of plates for imaging

- Cells should be plated according to the following layout, 4-5 days before the imaging day. The optical controls (dye and beads solution for flat field and point spread function) should be added to the plate on the day of imaging:
- Flat field solutions are prepared with Acid Blue 9 for 638nm, Coumarin 102 for 405nm, and Fluorescein for 488nm laser lines (see Fluorescent dye solutions for flat field correction SOP).
- Microspheres solution for channel alignment and point spread function inspection are prepared on the day of imaging (see Sub-resolution and focal check beads solution for alignment and PSF measurements SOP)
- 150 µL of each solution is added per well following the plate staining (see CellMask and NucBlue v1.0 SOP).

Table 1: Typical plate layout for the Image Acquisition Pipeline.

	4	5	6	7	8	9
C	AICS0	AICS0	AICS0	AICS0	AICS0	TL (PR-mTESR)
D	AICS12cl105	AICS12cl105	AICS12cl105	AICS12cl105	AICS12cl105	Acid blue
E	exp line	exp line	exp line	exp line	exp line	Coumarin
F	exp line	exp line	exp line	exp line	exp line	Fluorescein
						Microspheres

Preparation of spinning disk systems for image acquisition

System Initiation

1. Switch on all power bars.
2. Turn on computer last.
3. Open Zen Blue after all components have been activated and when prompted, do automated stage calibration.

4. Under Locate switch all lasers to the "ON" position. Allow lasers to warm up for a minimum of 15 minutes before imaging.
5. Ensure incubator is set to 37°C at least 1 hr before imaging: On the touch screen navigate to the incubation tab. Adjust temperature with scroll bar to 37°C.

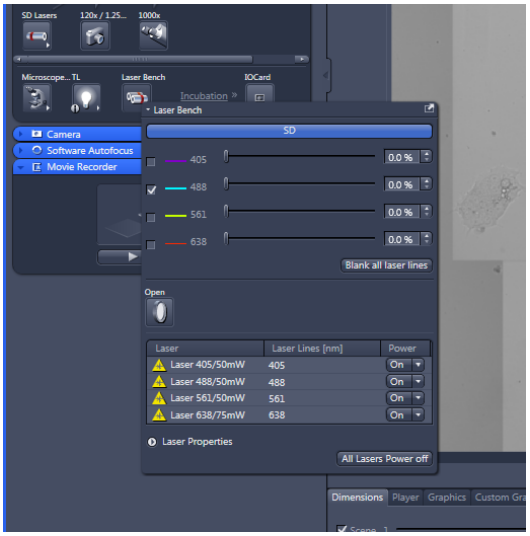


Figure 1 Switching laser to the "ON" position from the Locate Tab.

Daily maintenance

1. Ensure objective (10x and 100x) are clean.
2. Check 100x for collar correction adjustment (for 100x the red mark should be set to the 0.186 graduation – determined empirically).
3. Using gloves, clean with a wet lens paper the eye piece, condenser lens and stage optical lid (for 96-well plate insert).

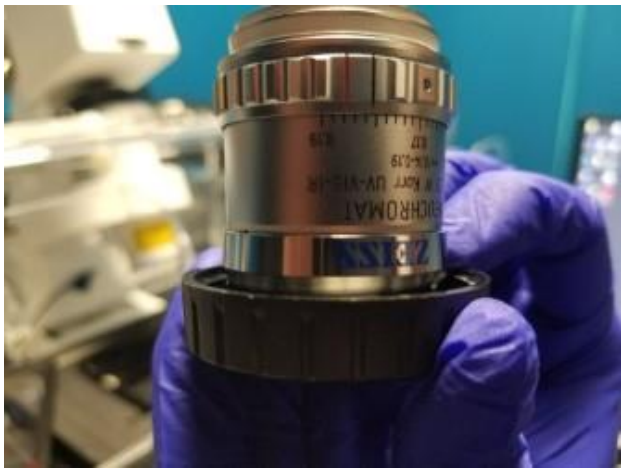


Figure 2 100x objective correction collar adjustment.

96-well plate calibration & Kholer illumination

1. Select acquisition tab. Select pipeline template experiment (eg. Pipeline4_delays_BR from the pull down menu at the top of the tab) and "save as" new experiment with the addition of today's date (eg. Pipeline4_delays_BR_20170915). This allows us to keep experiment set up for each acquisition, including the selected positions.
2. Place on the stage the 96-well plate to be imaged (keep plastic lid on if the cells are to be kept sterile, wipe the bottom of the plate with a wet lens paper).
3. To perform carrier calibration, select the Brightfield (BF) 10x channel in the "Channels Tab" area and focus on the bottom of the plate (either edge of a well or cells) using live view.
4. Under the "Tiles" tab, click on the Sample Carrier sub-heading to expand the menu. Click on "Calibrate" and follow the instructions of the sample carrier calibration wizard.
5. Select the “Search Well Edges (7 points)” methods. Follow the instructions as prompted. By clicking "move stage" between positions, the microscope will move close to where the next position should be. Make any minor adjustments to the position and adjust focus based on image, and confirm position by clicking highlighted blue circle at the well edge. (You can also move to the position using the joy stick).
6. Hit “Finish” at bottom of the screen when completed.

7. Set Kholer illumination by clicking on C6 (or any other well) under "Tiles" tab and drop down menu "Tile Regions". This will bring the field of view (FOV) directly in the center of the well.
8. Select "Live" to focus on a colony.
9. Close the Field Diaphragm.
10. Adjust the condenser position using the two black knobs until the edge of the Field Diaphragm is in focus.
11. Center the Field Diaphragm in the FOV.
12. Open the Field Diaphragm completely.
13. Adjust the Field aperture of the condenser under the Imaging set up tab. After selecting the appropriate track (i.e. either Bright 10x or Bright 100x) use the drop down menu of the bright field icon on the light path and adjust the N.A. to either 0.36 (10X) or 0.55 (100X). (This is set up as part of the experiment set-up, but it is a good idea to confirm.)

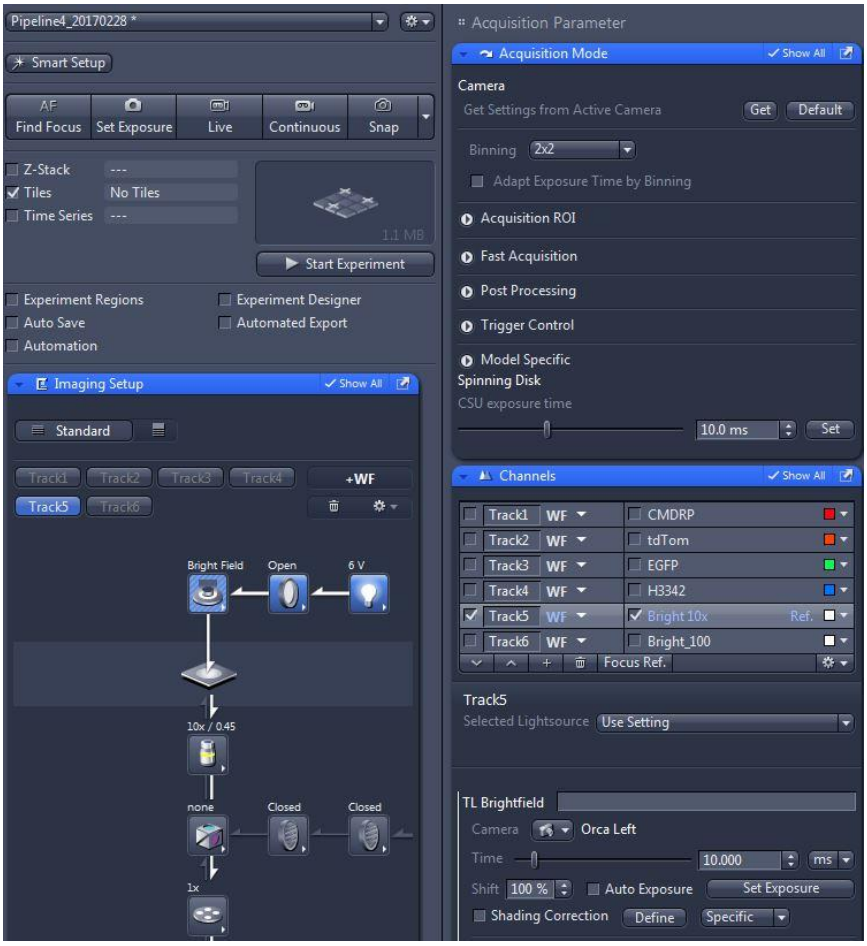
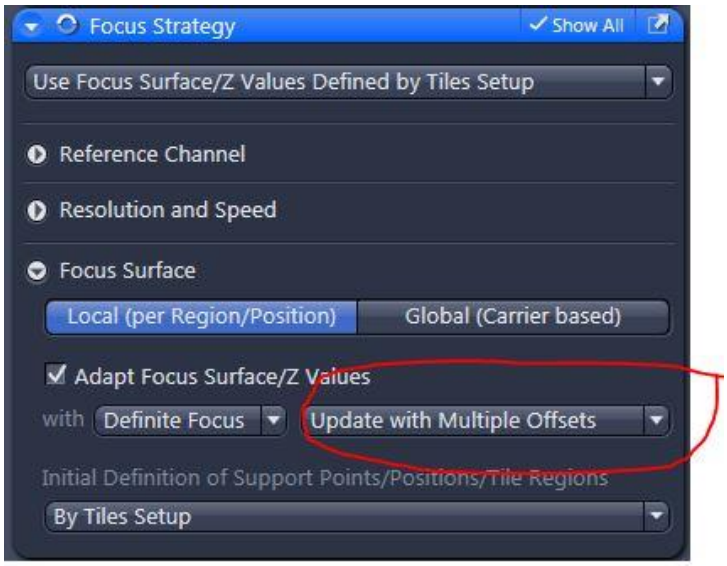
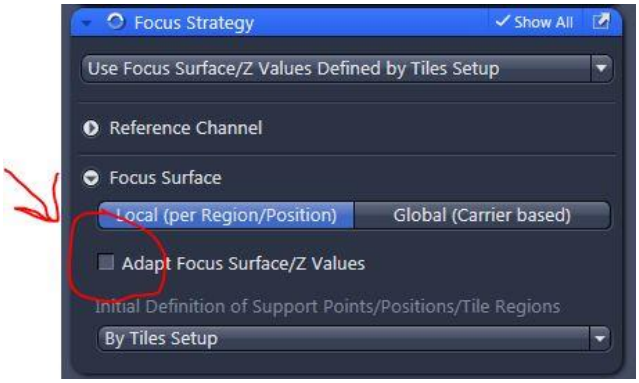


Figure 3 Image Setup is used to adjust the NA of the bright field aperture.

10x overview imaging of 96-well plates prior to staining

Table 2 Overview Imaging Settings

tab	item	position
Acquisition	experiment	Pipeline4_delays_BR_date
Acquisition	multi-	only tile should be checked
Imaging Set up	Bright_10X	NA should be set to 0.36 and checked, voltage should be set at ~5-6volt and checked in the Before Experiment; hardware set up (there are minor variations between the scopes in the lamp voltage).
Acquisition mode	binning	2X2
Acquisition ROI	FOV size and offset	FOV size for all systems: 1848 by 1248. **Offset: ZSD1=120 & 300, ZSD2=100 & 352, ZSD3=200 & 388,**
Channels	tracks	select Bright_10X only, adjust BF exposure time to 10ms, 0.36 NA and voltage to saturate approximately half of dynamic range for a whole well (approx. 6.5V) and 2x2 binning.
Focus Strategy	focus strategy for 10X	 Using Definite Focus 2.

		 If Definite Focus is not in use, uncheck Adapt Focus/Z Values.
Tile tab	Options	Tile Overlap should be set to 10%. **Make sure once again that the "split scene into separate files" option is unchecked.**

14. After Kholer Illumination is set, keep plate on spinning disk for well overview imaging at 10x (keep the plate lid on!!) before removing the plate for staining.
15. Review experiment settings (Table 2) and ensure all settings are correct for 10x overview imaging (these settings should be set by the experiment but should be checked nevertheless).

Specific Experiment Settings (10x)

1. Image one type of cell line at a time by selecting respective wells on carrier template and adding them as a tile region with a Fill Factor of 100% on the tile tab (these positions can be saved in an experiment, so most of the time will be already set up). Select the first well to be imaged in the tile region and with Live imaging, set up focus. Highlight and select wells to be imaged and right click to set all wells to the current Z position. The Z position can also be finely adjusted per well. Click "Start Experiment". All well overviews should be performed with Bright field only.
2. Save files for each cell line separately.
3. Plate Staining: return plate to incubator in tissue culture room for staining protocol to take place (see CellMask and NucBlue v1.0 SOP)

100x Z-stack position selection

This step is done using the overview images collected at 10x of each well, ideally before/during staining of the plate.

Well selection for 100x imaging (QC step):

1. **Use only wells with colonies of at least 100 cells and well packed**, (see WTC culture v1.1 and Cell plating for imaging v1.0 SOPs for references)
2. **Do not use wells that have been flagged for health reasons**:
    - Wells with balling colonies (>3 balling events/well),
    - Wells containing differentiation (>2 identifiable event/well)
    - Wells with high number of dead cells (>50% of cells/well)
    - Wells with less than 2 good positions (just not efficient and may be not enough colony or mature colony)**.**
3. **Do not image more than 10 positions per well** (due to phototoxicity effects) and beyond an hour post first exposure to laser insult (1 hr post first Z-stack acquisition in the imaged well).

Imaging Modes

Described below are the different modes of imaging which determine how positions of a Z-stack are selected within a colony.

Mode A

Uniform Area - mid-center (like mini-pipeline)

1. Choose 1 location per colony of mature size (>100 cells) that are nicely packed and flat. If colonies have merged, select an area within the merged colonies where single colony can still be distinguished by bridge formation between two colonies.
2. Avoid areas where there are dead cells on top of the colony when possible.
3. Avoid the ridge (taller cells along the edge of the colony.
4. Avoid colonies with lifted edge.
5. Avoid edges of colony.

Mode B

Uniform Area - mid-center (like mini-pipeline) + Mitotic enrichment

Same as Mode A plus the following additional steps:

1. Enrichment of the FOV for mitotic event
2. Performed at 100X just before acquisition of Z stack
3. Searching for mitotic event is done in LIVE mode using the BF channel

Mode C

Multiple areas within a single mature colony (triplet Z-stack per colony, each Z-stack with optimal height)

1. Choose 3 locations per colony (this will be performed on a maximum of 3 large and mature (>300 cells), nicely packed and flat colonies per well.)

2. Avoid areas where there are dead cells on top of the colony when possible.
3. Avoid colonies with lifted edge.
4. Select in sequence
   4.1. an edge position (short stack for minimal phototoxicity)
   4.2. a ridge position (Tallest Z stack to include the tall and thin cell shape)
   4.3. and a mid-center position (60-70 Z planes on average)

Select Individual Positions

Use the open overview tile scan and 10x objective.

To add positions:

1. Select Stage (1) under the "Dimension" tab for the image of the well targeted.
2. Click on a 10X overview image (the FOV should be large (a whole tile size) at 10X)
3. Using the touch screen pad select the 100x objective (no need to add water just yet). This should reduce the FOV to what it would be using the 100x (2).
   *****This order matters!!! Make sure to click stage, then change objective from 10X to 100X at the touchpad.**
4. Once an area has been selected on the image, the stage on the microscope will physically move to this position. Check that the stage is still reasonably positioned. Once the stage has moved, use the + arrow under the "Position" tab (3) to record the position.
5. Repeat steps 1-4 until 40-60 positions total have been recorded, using as many wells as necessary **(no more than 10 positions per well)**, while avoiding flagged wells.
6. In addition to colonies, select 1 area for background correction: one empty area (no cells), to be collected at the end of experiment, and one area for a Z-stack of the unlabeled control C0 cells (in C4-C8) - OPTIONAL. The AICS0 Z-stack should be acquired at the end of the imaging session.

   ***Sometimes switching between image files (ie. of the experimental cell overviews to the AICS0 overviews) can cause stage problems; add the AICS0 position from overview images using caution. To avoid issues, it is best to click on "Stage" function while still using the 10X objective and then click over the AICS0 overview images and follow steps 2-4 above as for the experimental position selection.

7. Ensure the Z plane of the selected position is comparable to the Z plane of the tile regions (it should be approximately 9300-9600 µm if using the Okolab holder). If not double click on a tile region, select all positions (all P), right click and select "set current z for selected position" after selection for all position is completed.
8. **Save today's Experiment one last time before proceeding to the next Step**.

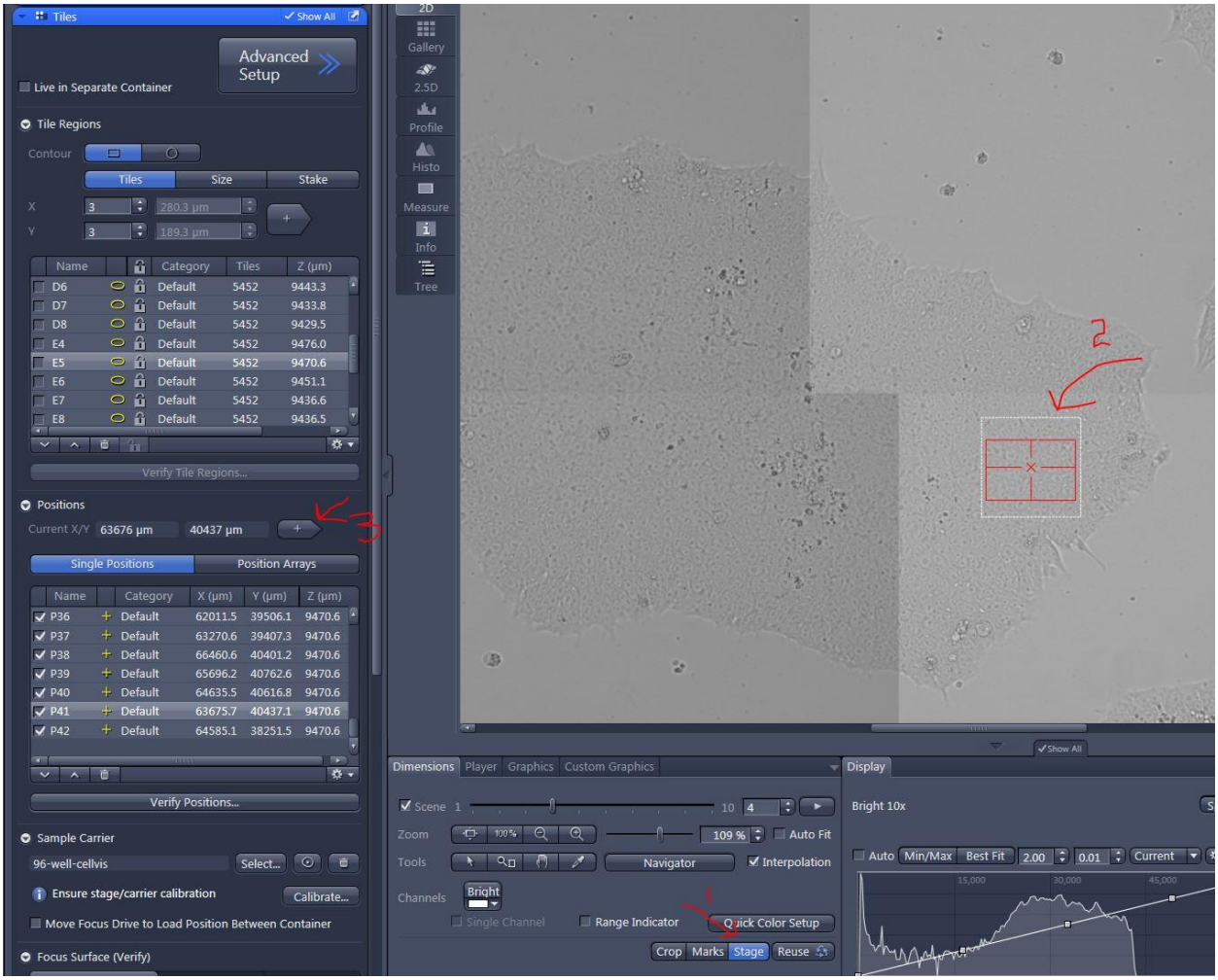


Figure 4 Position selection using the stage function.

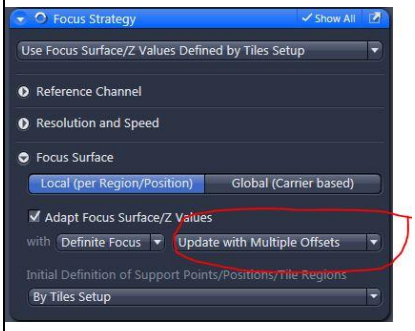
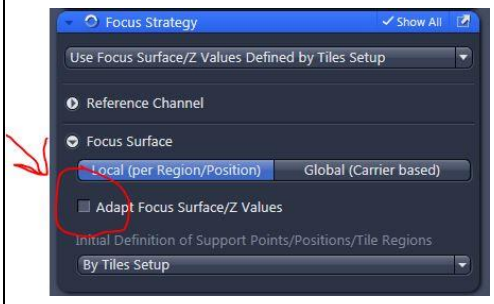
tab	item	position
Acquisition	multi-	Z-Stack and tile should be checked
Imaging Set up	Bright Field	NA should be set to 0.55 and checked in the Before Experiment; hardware set up and voltage should be set to 10volt.
Acquisition mode	binning	2X2
Acquisition ROI	FOV size and offset	FOV size for all systems: 1848 by 1248. **Offset: ZSD1=120 & 300, ZSD2=100 & 352, ZSD3=200 & 388,**
Channels	tracks	select the following channels CMDRP (638nm), EGFP (488nm), H3342 (405nm) and Bright Field, adjust BF exposure time to 100ms and all other channels to 200ms (*250 ms exposure time for the 405 nm laser on ZSD3). Laser power should be adjusted to the previously determined laser power at objective (see Laser power measurement SOP): **405=0.28mW** **488=2.3mW** **561=2.4mW** **638=2.4mW**
Focus Strategy	Focus strategy for 100X	 Using Definite Focus 2.  If Definite Focus is not in use uncheck Adapt Focus/Z Values.
tile tab	Options	make sure once again that the “split scene into separate files” option is unchecked.

Table 3 Z-Stack Imaging Settings

High-resolution imaging following staining with CMDR and Hoechst

1. Following the completion of the staining protocol (start timer after last wash) bring plate back to spinning disk for 100x imaging.
2. Make adjustments as described in Table 3 if needed to adjust imaging parameters for 100x, multi-channel acquisition if necessary.
3. Select Bright_100x channel for transmitted light.

Specific Experiment Settings (100x)

Definite Focus Workflow

1. Set the number of Z slices to be acquired next to slices under Z-stack tab; for most cell lines when imaging well-packed center colony regions, 70 z-stacks is a reasonable number of slices, although some cell lines may be thicker or thinner.
2. Visit each single position to be acquired and make sure that the x-y position is good (e. well

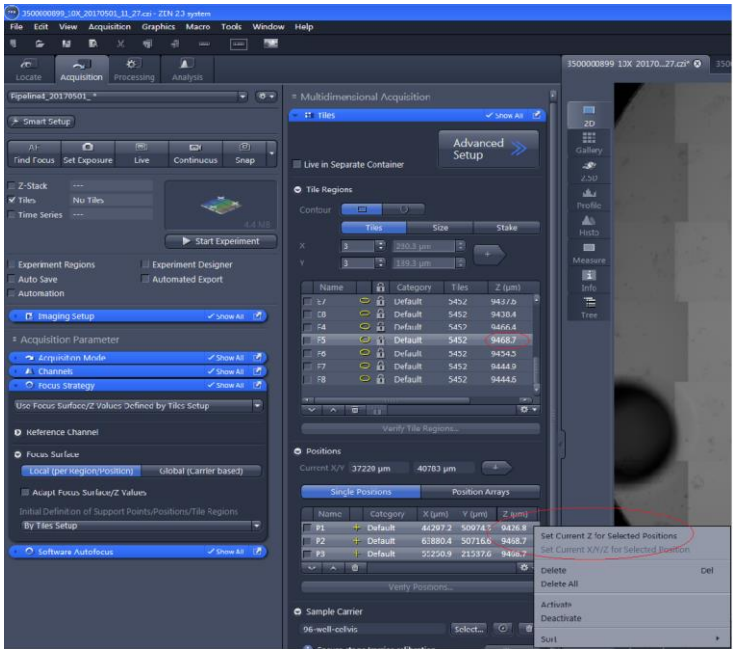
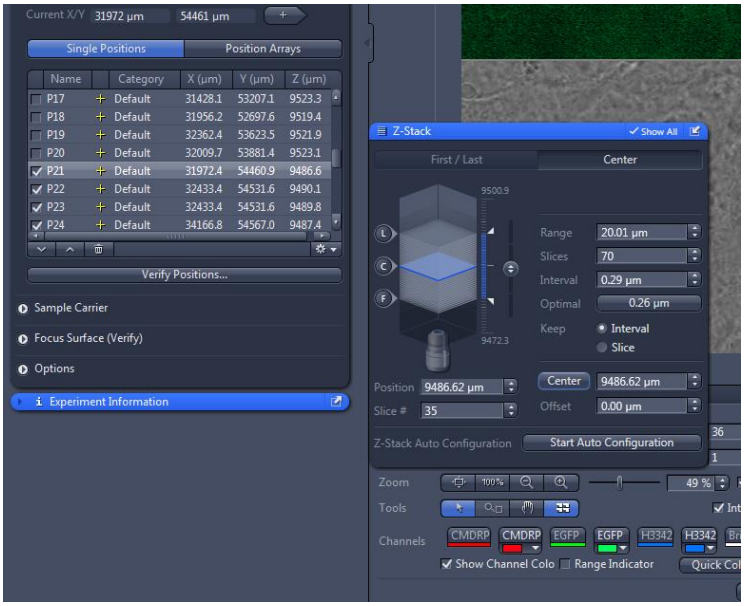


Figure 5 Z position adjustment.

packed cells, no dead debris on top). Adjust the x-y position and adjust to the center focal plane, then right-click on the position and click "Set Current XYZ for Selected Position".

2. If using definite focus, set up your focus strategy as shown above. You will also need to switch to the center tab of the Z stack menu. Before proceeding make sure that the first single position is set to the center plane shown on the Z-stack menu (Figure 6), (select first position in tile tab/ Position, right mouse click and select "set new x,y,z position").

Figure 6 Z stack tab settings



2.1. Start your experiment (1). The "Prepare Definite Focus Initialization" window will pop up once you start the experiment.
2.2. Click on "Manual Determination of Z values and offsets" drop down menu (2).
2.3. Open "LIVE" Image using BF channel (Top menu).
2.4. Click "Move to current Point" (3)
2.5. Adjust focus and click on "Set Z & Move to Next" (4) (Figure 7)
2.6. Repeat until the last position has been checked.
2.7. Check that the laser safety lights (small button box next to the microscope base) are "on".

2.8. Press Continue Experiment (which should now be enabled; 5)

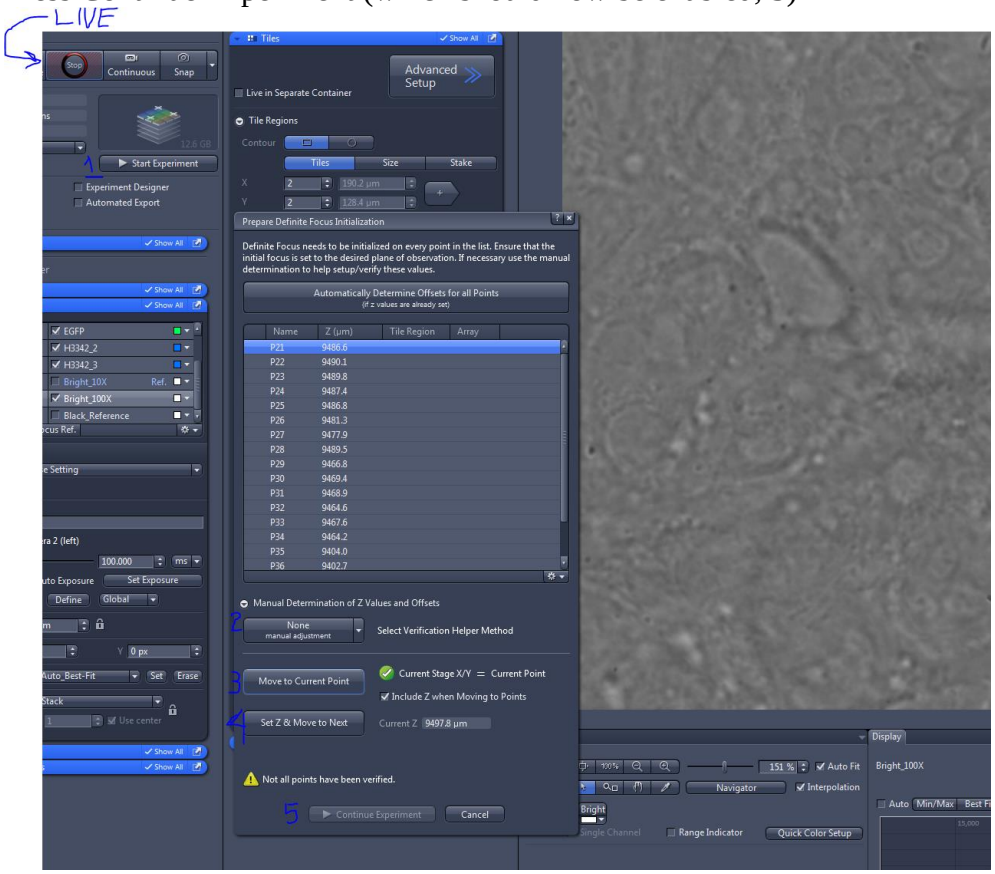


Figure 7Manual determination of Z-values and Offsets

Manual (no definite focus) workflow

1. Set "Focus Strategy" tab as shown for Definite Focus not in use above.
2. With the transmitted light channel highlighted, click "Live" and one by one, visit previously identified positions, adjust z-position for each colony using Z stack menu. Under the First/Last tab determine top and bottom Z plane of the cells/colony, hit the "c" and save the new z position in tile tab/ Position (right mouse click and select "Update XYZ position").
3. Check that the laser safety lights (small button box next to the microscope base) are "on".
4. Start experiment with a single position checked (no definite focus).

Measure the CNR (contrast to noise ratio) for Hoechst at the beginning of acquisition on the first 1-2 images, and for the CellMask Deep Red every ten images or so. Imaging of cells should be completed within 2-2.5 hours of final wash step of staining protocol (or until CNR is below the acceptable levels, but this is rare on the pipeline systems with binning).

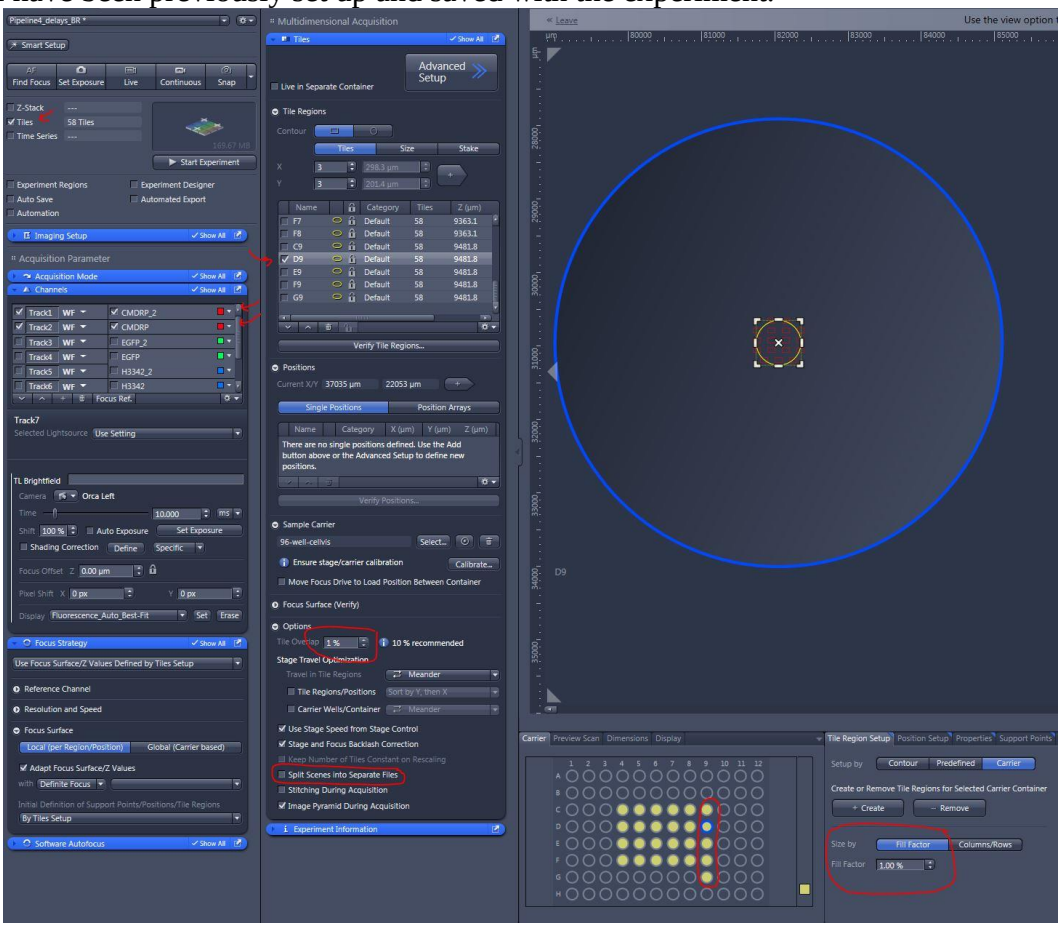
Imaging of optical controls

Purpose

Optical controls are acquired for each imaging plate and are used for daily quality control of system performance as well as for image correction and normalization.

Acquisition

1. Imaging of flat-fielding (dye) controls (still using 100x):
2. Using Advanced Setup, select one well containing the flat-fielding samples at a time using the template (C9 =empty for TL, D9=Acid Blue for 638 (red), E9=Coumarin for 405 (blue), F9=Fluorescein for 488(green).
3. Using the "Tile Region Setup" Tab select carrier at the bottom of the screen, then change the Fill Factor to 0.97% and press create (Figure 8).
   *These positions can be saved as part of an experiment, so most of the time these positions will have been previously set up and saved with the experiment.



i. Figure 8 Tile distribution within a single wells for flat fielding image acquisition.

4. Do not use definite focus for flat field dye wells. Instead, set focus strategy to “absolute fixed z position” and set the exposure time to **100ms** for TL, **200ms** for 638 and 488 and to **250ms** for 405. Unselect Z-stack and any other Tile regions or Single positions.
5. Adjust the focal plane by defocusing just slightly into the solution to reach max peak intensity on the display histogram. The goal is to have a uniform fluorescent area without seeing debris or interference from the matrigel. The intensity should be of at least a 1000 grey levels on the display histogram.
6. Under Tiles ensure that under Options the "Tile Overlap" is set to 1% and "Split Scene” is unchecked.
7. Take flat-fielding images of each channel/well combination individually (i.e. Select single well and respective single channels) and save all files individually.
8. Collect bead images; two stacks in total.
   8.1. Identify an area rich in larger focal check beads by scanning around the well. Collect a z-stack through the entire volume, using all fluorescent channels (30 Z planes).
   8.2. Adjust exposure time to 500ms for all channels, unselect Tiles, and make sure focus strategy is set to none.
   8.3. Navigate in an area with no large bead in the center of the FOV where at least 10-20 sub-resolution beads are visible.Take a z-stack through the sub-res beads (30 Z planes).
   8.4. Save files.
9. Collect Black reference.
   9.1. Select Black_Reference channel. (uses non as filter and 100%VIS, all TL shutter close and 0 laser power.)
   9.2. Select only Time Series (50 cycles).
   9.3. Acquire black reference with 200ms exposure time.
   9.4. Save file.

★ Cool down system before turning it off.

Adjust incubator setting to 24°C and allow the system to cool off before it is shut down: On the touch screen navigate to the incubation tab. Adjust temperature with scroll. Turn system off when temperature reaches ~25°C.

Batch Process Multi-scenes data sets (Split Scenes from the 100x image acquired with definite focus or using multi-positions)

1.1. Use the Processing Tab
1.2. Select Function: Batch
1.3. Drag original files (non-split scenes) into the Batch Processing Window in Zen Blue.
1.4. Under “Batch Method” tab, select “Split Scenes”.
1.5. Under Parameters tab, identify the path where the new split files should be.
1.6. Check “Include Scene Information in Generated File Name”.
1.7. In the Batch Processing window, highlight the first file.
1.8. Click on Copy Parameters
1.9. Select all files in the Batch Processing Window

1.10. Click on Paste Parameters
1.11. Click on Check all
1.12. Click on Apply

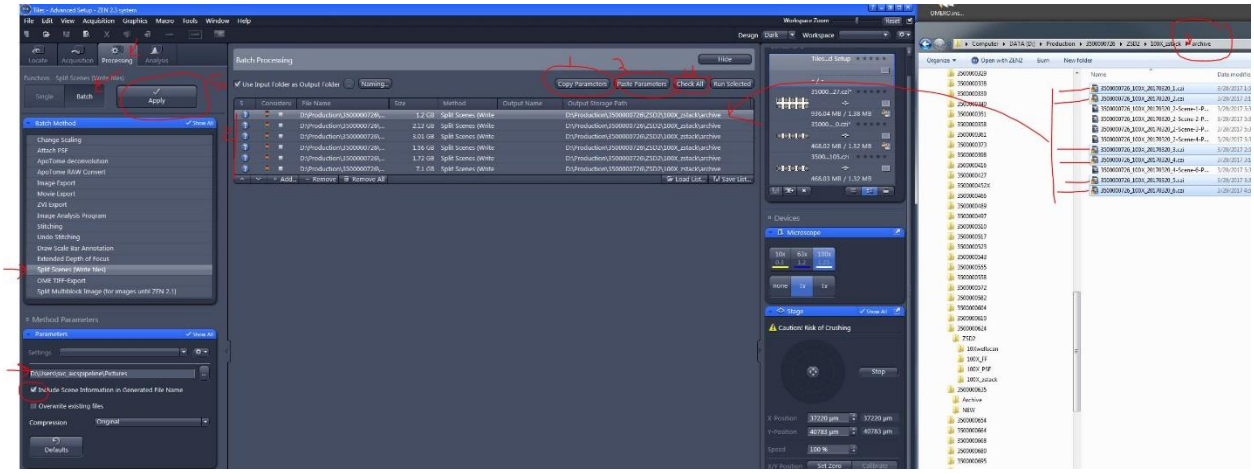


Figure 9 Batch Processing for scene spitting.

Data Review and curation

1. Ensure FF have at least a 1000 count of white level in Intensity. See examples below showing typical flat field with color of LUT/Rainbow display:

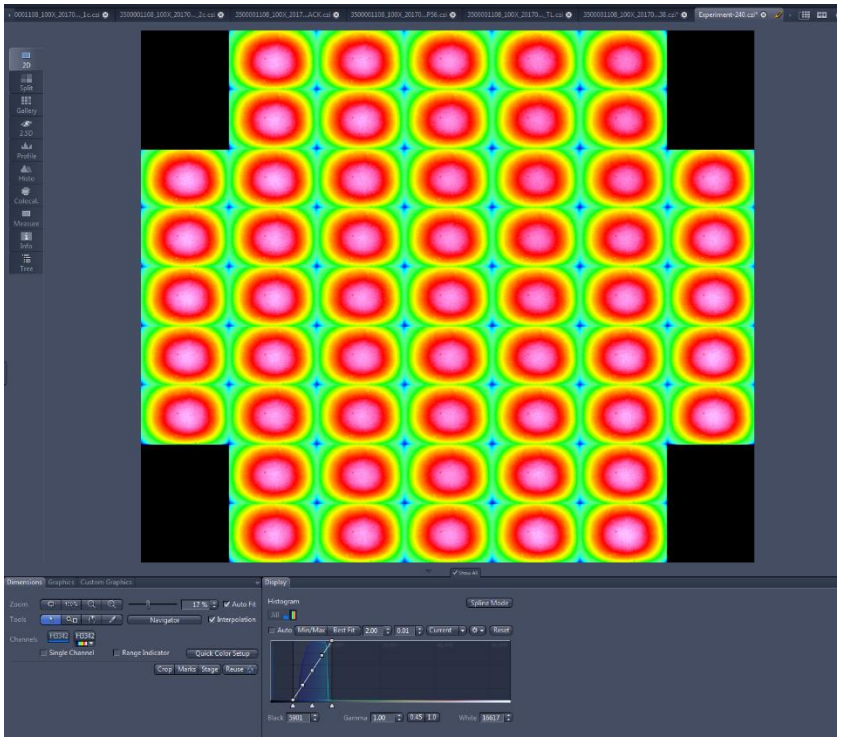


Figure 10 Example of Rainbow LUT view of tiled single plane of coumarin solution collected with the 405 laser line.

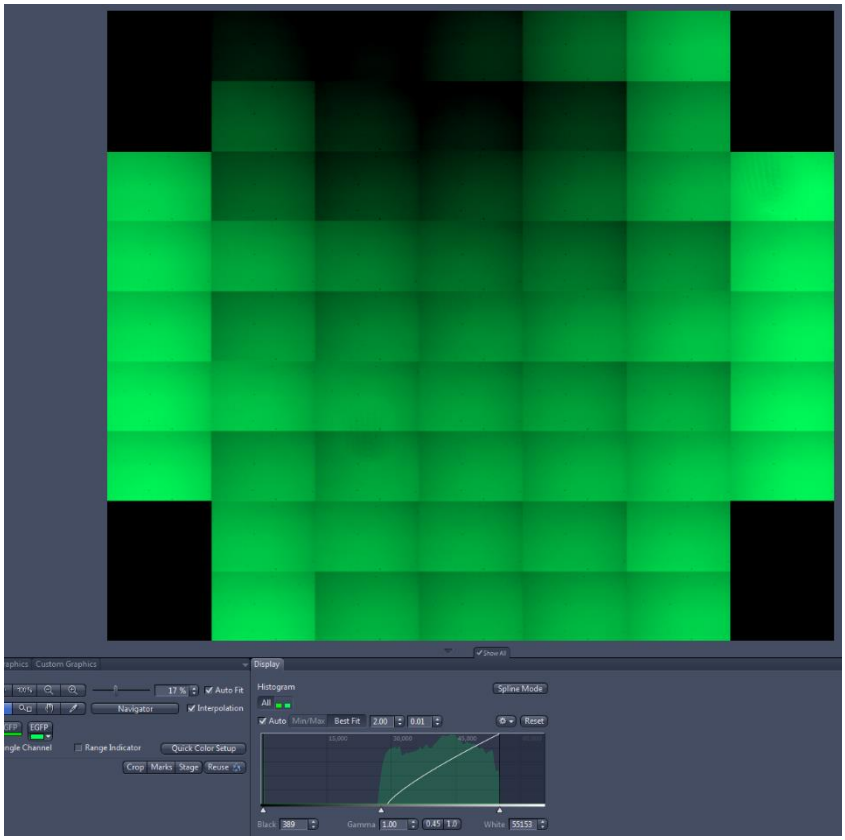


Figure 11 Example of unicolor view of tiled single plane of fluorescein solution collected with the 488 laser line.

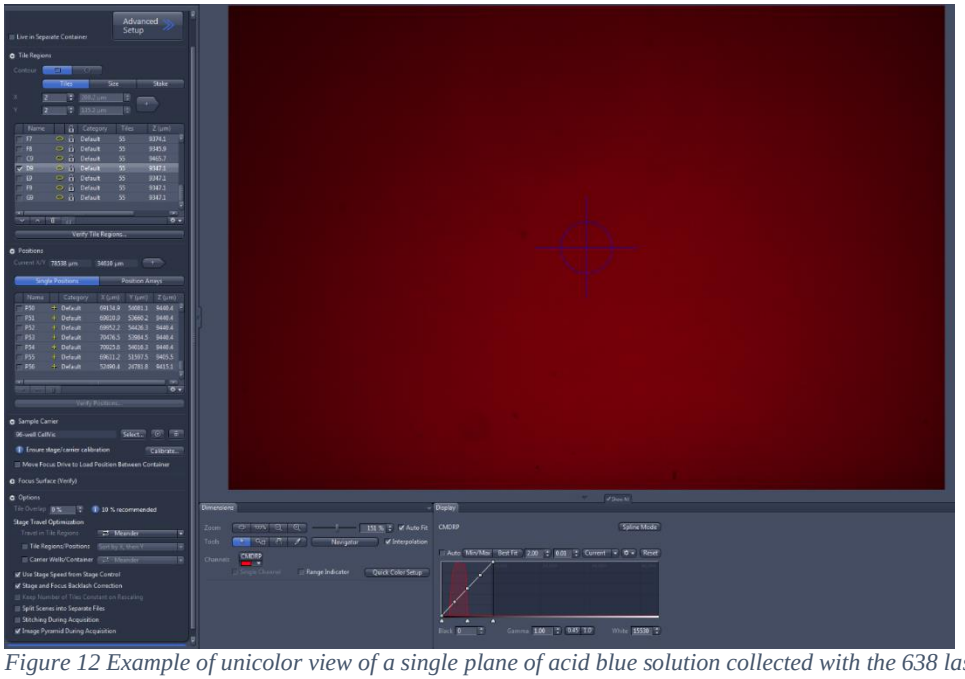


Figure 12 Example of unicolor view of a single plane of acid blue solution collected with the 638 laser line.

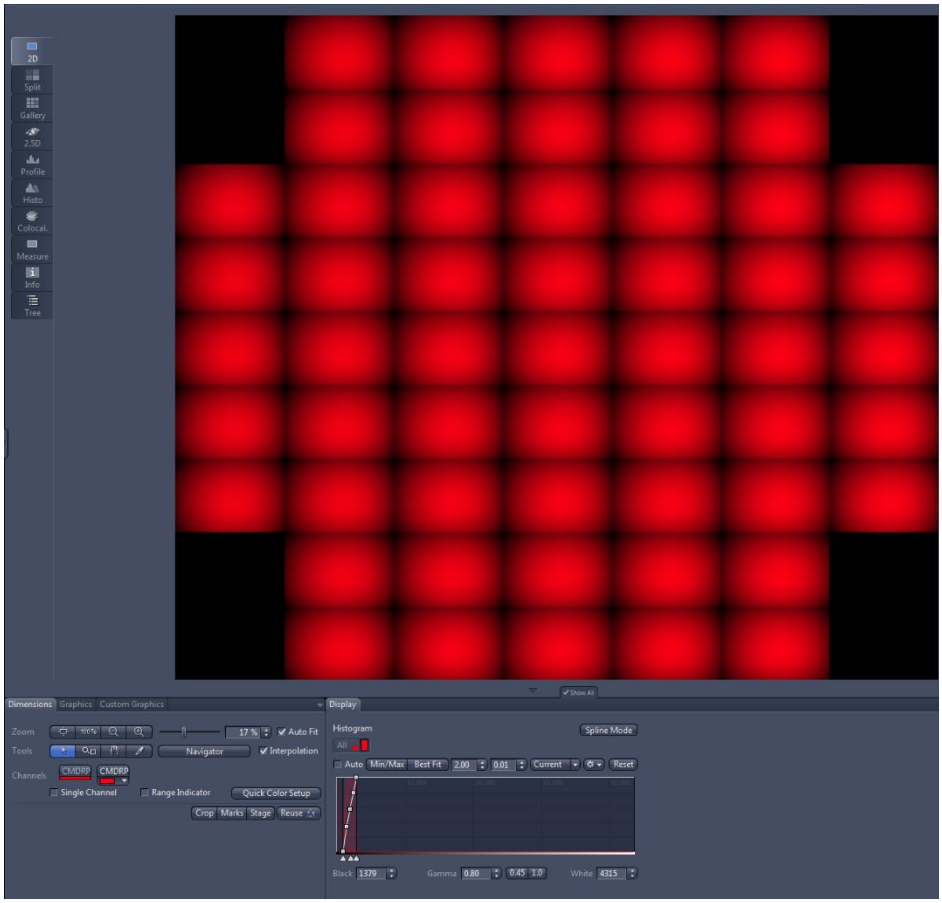


Figure 13 Example of unicolor view of tiled single plane of acid blue solution collected with the 638 laser line.

2. Ensure large beads (>4µm) images from the different channels are aligned within 2 pixels.
3. Ensure PSF are symmetrical and perpendicular to the Z plane.
4. Report any discrepancy to the team and indicate in the note text file and comment in the data log.
5. Review each Z stack by dragging multi-scenes or split scene into Zen Blue (under Acquisition Tab or Analysis Tab).
6. Look through the data and identify and exclude Z stacks with:
   6.1. cropped top or bottom (more than a quarter of the FOV is cropped on on side)
   6.2. Larger number of dead cells within or on top of the colony
   6.3. Poor cell packing with large gaps between cells (except for ridge areas)
   6.4. Imaging triggered cell death (blebbing, filapodia retraction)
   6.5. Missing data or unexplained movement (bumps or vibration)
7. Move identified failed scenes to an Archive folder. These should not be processed for segmentation at this point.
8. Now you're done!!!