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Live-cell forward genetic approach to identify and isolate developmental mutants in Chlamydia trachomatis.

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Cover Letter

Dear Jove editorial board,

Thank you for considering our manuscript entitled "Live-cell forward genetic approach to identify and isolate developmental mutants in *Chlamydia trachomatis*. " This article describes a novel forward genetic technique to identify genetic determinants of the chlamydial developmental cycle. We leverage chlamydial exenic metabolism, cell type promoter reporters, live cell imaging and novel analysis and visualization techniques to isolate phenotypic developmental mutants. Please do not hesitate to contact us for more information.

Best,
Scott Grieshaber

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1   **TITLE:**
2   Live-Cell Forward Genetic Approach to Identify and Isolate Developmental Mutants in *Chlamydia*
3   *trachomatis*
4
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17   **KEYWORDS:**
18   Forward genetics, chemical mutagenesis, chlamydial development, live-cell microscopy,
19   automated microscopy, fluorescent reporters.
20

21   **SUMMARY:**
22   This protocol utilizes fluorescent promoter-reporters, live-cell microscopy, and individual
23   inclusion extraction in a directed forward genetic approach to identify and isolate developmental
24   mutants of *Chlamydia trachomatis*.
25

26   **ABSTRACT:**
27   The intracellular bacterial pathogen *Chlamydia trachomatis* undergoes a developmental cycle
28   consisting of two morphologically discrete developmental forms. The non-replicative elementary
29   body (EB) initiates infection of the host. Once inside, the EB differentiates into the reticulate body
30   (RB). The RB then undergoes multiple rounds of replication, before differentiating back to the
31   infectious EB form. This cycle is essential for chlamydial survival as failure to switch between cell
32   types prevents either host invasion or replication.
33

34   Limitations in genetic techniques due to the obligate intracellular nature of *Chlamydia* have
35   hampered identification of the molecular mechanisms involved in the cell-type development. We
36   designed a novel dual promoter-reporter plasmid system that, in conjunction with live-cell
37   microscopy, allows for the visualization of cell type switching in real time. To identify genes
38   involved in the regulation of cell-type development, the live-cell promoter-reporter system was
39   leveraged for the development of a forward genetic approach by combining chemical
40   mutagenesis of the dual reporter strain, imaging and tracking of *Chlamydia* with altered
41   developmental kinetics, followed by clonal isolation of mutants. This forward genetic workflow
42   is a flexible tool that can be modified for directed interrogation into a wide range of genetic
43   pathways.
44

INTRODUCTION:

Chlamydia trachomatis (Ctr) is an obligate intracellular pathogen that progresses through a biphasic developmental cycle that is essential for its survival and proliferation[1]. This cycle consists of two developmental forms, the elementary body (EB) and the reticulate body (RB). The EB is replication incompetent but mediates cell invasion through effector induced endocytosis[2]. Once in the host, the EB matures to the replicative RB. The RB carries out multiple rounds of replication prior to converting back to the EB in order to initiate subsequent rounds of infection.

The limited array of genetic tools has restricted most of the chlamydial research to biochemical studies or the use of surrogate systems. As a consequence, elucidation of gene regulation and control of the developmental cycle has been difficult[3,4]. One of the more important challenges in the chlamydial field is the high resolution temporal tracking of the chlamydial developmental cycle and the identification of the proteins involved in its regulation. Gene expression during the chlamydial developmental cycle has traditionally been performed by destructive "end point" methods including RNAseq, qPCR, and fixed cell microscopy[5,6]. Although these methods have provided invaluable information, the techniques employed are laborious and have low temporal resolution[5,6].

Within the last decade, genetic manipulation of Ctr has progressed with the introduction of plasmid transformation and methods for mutagenesis[7-9]. For this study, a plasmid-based system was developed to monitor chlamydial development in individual inclusions in real time over the course of an infection. A chlamydial transformant was created that expressed both an RB and EB cell-type specific promoter-reporter. The RB specific reporter was constructed by fusing the promoter of the early RB gene *euo* upstream of the fluorescent protein Clover. EUO is a transcriptional regulator that represses a subset of late EB associated genes[10]. The promoter of *hctB*, which encodes a histone-like protein involved in EB nucleoid condensation, was cloned directly upstream of mKate2 (RFP) to create the EB specific reporter[11]. The backbone for *hctB*prom-mKate2/*euo*prom-Clover was p2TK2SW2[7]. The *hctB* and *euo* promoters were amplified from Ctr-L2 genomic DNA. Each promoter sequence consisted of ~100 base pairs upstream of the predicted transcription start site for the specified chlamydial gene plus the first 30 nucleotide (10 amino acids) of the respective ORF. The fluorescent FP variants were commercially obtained as Ctr codon optimized gene blocks and cloned in frame with the first 30 nucleotide of each chlamydial gene and promoter. The incD terminator was cloned directly downstream of mKate2. The second promoter-reporter was inserted downstream of the incD terminator. The ampicillin resistance gene (*bla*) in p2TK2SW2 was replaced with the *aadA* gene (Spectinomycin resistance) from pBam4. This resulted in the final construct p2TK2-*hctB*prom-mKate2/*euo*prom-Clover (**Figure 1A**) that was transformed into Ctr-L2[7]. This RB/EB reporter strain allowed for the observation of the developmental cycle within single inclusions using live-cell microscopy (**Figure 1B,C**).

Employing our promoter-reporter construct in combination with chemical mutagenesis, a protocol was devised to track and isolate individual clones that exhibited developmental abnormalities from mutagenized populations of Ctr serovar L2. This protocol allows for the direct monitoring of individual chlamydial inclusions, tracking of the gene expression profiles over time,

89   identifying chlamydial clones that express an altered developmental gene expression pattern,
90   and clonal isolation of *Chlamydia* from individual inclusions.
91
92   Although this protocol has been created specifically for the identification of genes involved in
93   chlamydial development, it could be easily adapted to interrogate any number of chlamydial
94   genetic pathways.
95
96   **PROTOCOL:**
97   All Python scripts used in this protocol are available on Github
98   https://github.com/SGrasshopper/Live-cell-data-processing
99
100   **1. Mutagenize Reporter *Chlamydia***
101
102   NOTE: Ctr-L2-*hctB*prom-mKate2/*euo*prom-Clover EBs were directly mutagenized using ethyl
103   methanesulfonate (EMS) in the axenic media CIP-1 as this media supports EB metabolism and
104   maintenance of EB infectivity[12].
105
106   1.1. Thaw a chlamydial stock on ice containing ~3 x 10^7 EBs transformed with the p2TK2-
107   *hctB*prom-mKate2/*euo*prom-Clover reporter plasmid and pellet at >14,000 *x g* for 30 min at 4 ˚C.
108
109   NOTE: *Chlamydia* organisms used for these experiments were 30% renografin density purified
110   and frozen at -80 °C in 1x sucrose-phosphate-glutamate buffer (SPG).
111
112   1.2. Discard the supernatant and resuspend the EB pellet in 100 μL of CIP-1 buffer with sonication
113   on ice at 10% power for 10 s. Divide the 100 μL of EB suspension into two 50 μL aliquots for
114   mutagenized and mock treated samples.
115
116   1.3. Prepare 20 mg/mL of EMS-CIP-1 solution in a separate 1.5 mL microcentrifuge tube. To do
117   so, add 6.8 μL of EMS in 375 μL total volume.
118
119   1.4. Add 50 μL of the EMS-CIP-1 solution into one of the chlamydial aliquots for mutagenesis and
120   50 μL of CIP-1 only to the other chlamydial aliquot for mock mutagenesis.
121
122   NOTE: Final EMS concentration is 10 mg/mL. The chlamydial titer, EMS concentration, and the
123   time of exposure used in this protocol lead to approximately a 60-80% reduction in infectious
124   progeny. This level of reduction corresponds to ~5-20 DNA lesions per chlamydial genome[8].
125
126   1.5. Incubate for 20 min at room temperature. The mutagenized EBs will be used directly to infect
127   monolayers in section 2.
128
129   CAUTION: EMS is a known carcinogen. All equipment and materials that come in contact with
130   EMS must be soaked in 1 M NaOH for 24 h before disposal, gloves should be used at all times
131   during the protocol and cleanup of EMS materials.
132

2. Imaging of mutant Ctr

2.1. Host cell culture for imaging and isolation of mutagenized Ctr

2.1.1. Seed a 6 well glass bottom plate with 6×10^5 Cos-7 cells (ATCC) per well in 2 mL of complete media (RPMI-1640 supplemented with 10% fetal bovine serum and 10 mg/mL gentamicin). Use this glass bottom plate for imaging of mutagenized Ctr.

2.1.2. Seed a 24 well polystyrene plate with 1×10^5 Cos-7 cells (ATCC) per well in 1 mL complete media. Use this polystyrene plate for reinfection of isolated *Chlamydia* of interest.

2.1.3. Incubate both the plates at 5% CO_2, 37 °C for approximately 18 h. Once cells reach confluency, replace media with complete media supplemented with 1 μg/mL of cycloheximide and incubate overnight.

2.2. Infecting the host cell culture with mutagenized Ctr

2.2.1. Infect 5 wells of the glass bottom plate with ~6×10^5 of mutagenized EBs in 1.5 mL/well ice cold HBSS. This will result in the MOI of ~ 0.3 as ~70% mortality rate is expected due to mutagenesis.

2.2.2. Infect the remaining well with ~2×10^5 mock mutagenized EBs in 1.5 mL/well ice cold HBSS. Without mutagenesis, expect less mortality, thus one-third of the inoculum is used to achieve the MOI of ~0.3.

NOTE: MOI of ~0.3 ensures that host cells are infected by a single EB and allows for the enough separation between infected cells for clonal isolation.

2.2.3. Incubate the plate for 15 min, with rocking, at 37 °C.

2.2.4. Wash the infected host cells with prewarmed (37 °C) HBSS containing 1 mg/mL heparin followed immediately by an HBSS rinse. Repeat heparin wash, immediately rinsing 2x with HBSS to ensure the heparin is removed.

NOTE: Heparin inhibits and can reverse the early electrostatic interactions between the host cell and EBs[13]. The heparin washes remove EBs that have yet to enter the host cells, synchronizing the infection. When washing cells do so gently to prevent dislodging the cells from the surface of the wells. HBSS and heparin solutions contain residual EMS and should be placed in a beaker containing 1 M NaOH for 24 h before disposal.

2.2.5. Replace HBSS with 4 mL/well of prewarmed (37 °C) imaging media (complete media, 1 μg/mL cycloheximide, 20 mM HEPES, and no phenol red).

176 2.2.6. Fill the interwell spaces with prewarmed (37 ˚C) deionized H_2O to aid in the temperature
177 control and reduce evaporation. Incubate the plate at 37 °C incubator with 5% CO_2 for 10 h.
178
179 2.3. Microscope set up and imaging
180
181 NOTE: Multicolor multiposition automated live-cell fluorescent imaging is used to collect time-
182 lapse images to identify chlamydial mutants that differ in the developmental gene expression
183 dynamics. This protocol utilizes the open source µManager software package for automated
184 microscope control[14].
185
186 2.3.1. Begin the microscope setup 10 h post infection. Set the microscope stage incubator to 5%
187 CO_2, 37 ˚C. Place the infected 6 well glass bottom plate into the stage incubator and insert the
188 sample thermistor into the interwell H_2O.
189
190 2.3.2. Calibrate the XY stage using the High Content Screening (**HCS**) plugin. Click **Plugins |**
191 **Acquisition Tools | HCS Site Generator** in the µManager microscope control software
192 (**JoVE61365_screenfile1, JoVE61365_screenfile2**).
193
194 2.3.3. Select the 6-well plate template and generate an imaging position list consisting of 12 fields
195 of view (FOV) per well within the **HCS** plugin (**JoVE61365_screenfile3, JoVE61365_screenfile4**).
196 Open the **Stage Position List** and manually focus and set the initial Z position for each FOV using
197 the Stage Control plugin (**JoVE61365_screenfile5, JoVE61365_screenfile6**). Adjust the XY
198 coordinates of any FOV that has missing cells or does not contain a uniform monolayer.
199
200 NOTE: Due to the time it takes for each image to be captured, a maximum number of 72 FOV can
201 be taken per 30 min interval.
202
203 2.3.4. Use a 20x objective lens for imaging. This magnification allows ~8 inclusions to be imaged
204 per FOV while still providing the desired resolution.
205
206 2.3.5. Save the positions list as this will be used to locate the inclusions of interest after data
207 analysis (**JoVE61365_screenfile7**).
208
209 2.3.6. Use the **Auto Focus** option in the imaging software, to set the focus for automated imaging
210 (**JoVE61365_screenfile8**).
211
212 2.3.7. Use the following selections and values to produce the most consistent focus results using
213 image based autofocus in µManager. In the Autofocus properties window select **OughtaFocus**
214 from the drop-down menu and use the following settings. OughtaFocus-SearchRange_µm: 350,
215 OughtaFocus-Tolerance_µm: 0.5, OughtaFocusCropFactor: 0.3, OughtaFocus-Exposure: 20,
216 OughtaFocus-FFTLowerCutoff(%): 2.5, OughtaFocus-FFTUpperCutoff(%): 14, OughtaFocus-
217 ShowImages: Yes, OughtaFocus-Maximize: SharpEdges, OughtaFocus-Channel: DIC.
218

219 NOTE: Reducing the autofocus imaging window by decreasing the crop factor allows for more
220 consistent auto focusing. Selecting **Yes** for OughtaFocus-ShowImages allows the user to view the
221 autofocus image.

223 2.3.8. Capture the kinetics of the developmental cycle by imaging for 24 h with 30 min time
224 intervals (**JoVE61365_screenfile9**). Imaging between 12-36 HPI ensures that *Chlamydia*
225 completes the developmental cycle but does not lyse the host cell.

227 2.3.9. Image the cell monolayers with a 250 ms exposure at 4% and 18% intensity in the GFP and
228 RFP channels, respectively (**JoVE61365_screenfile10**). Detect the Clover (GFP) signal by excitation
229 at 470 nm with a 514/30 nm bandpass emissions filter. Detect the mKate2 (RFP) signal by
230 excitation at 595 nm and with a 641/75 nm bandpass emissions filter.

232 NOTE: Minimizing the excitation intensity is critical for minimizing fluorophore photobleaching
233 and phototoxicity to *Chlamydia*. The minimum excitation intensity to generate a resolved image
234 with a 200-300 ms exposure should be determined empirically in pilot studies.

236 2.3.10. Capture multiple Z-slices with a range of focus that ends on either side of the in-focus
237 slice. In this experiment, at 20x magnification, this was achieved with 4 slices at 10 μm steps
238 (**JoVE61365_screenfile11**).

240 2.3.11. Select **Relative Z** for imaging multiple slices in the acquisition window. The relative Z
241 option uses the Z plane location saved in the imaging position list as the starting point for the
242 next time interval. Input the appropriate Z-offset values for fluorescence imaging channels
243 (**JoVE61365_screenfile12**).

245 NOTE: The image based focusing system is imperfect and over a 24 h imaging period this leads to
246 focus drift. It was found that by capturing 3-4 Z focal planes for each time point an in-focus image
247 was maintained. Z-offset is needed to correct for the differences in focal planes between the
248 fluorescent image channels and the DIC channel. Empiric determination of this Z-offset will be
249 needed.

251 2.3.12. Save images by selecting the root directory and naming the experiment. Use the
252 μManager **image stack file** option to save images as tiff stack files (**JoVE61365_screenfile13**).

254 2.3.13. Record the experimental details in the **Acquisitions Comments** box in the Multi-D
255 Acquisition window (**JoVE61365_screenfile13**). i.e., Well A1: Untreated control. Well A2-3 and
256 B1-3: EMS Mutants. Imaging: 12-36HPI. Start the image acquisition at 12 HPI.

258 NOTE: If using μManager, leave the program, experimental setup, and microscope hardware
259 running after the experiment is complete. The imaging sites will be revisited for inclusion isolation
260 once analysis of the mutagenized population is completed.

262 **3. Identify and isolate mutagenized *Chlamydia* with altered developmental phenotypes**

263

264　3.1. Creating an in-focus image stack

265

266　3.1.1. Extract the most in-focus image from the Z-stacks using the saved image data which
267　contains 4 Z slices per time point. Use the kurtosis measurement option (**Analyze | Measure**) in
268　ImageJ/FIJI to automatically identify the most in focus Z slice (highest kurtosis score) and create
269　a new image stack with just these in-focus images. A Python script is included as a supplementary
270　file (**Reduce_Z_kertosis_2ch_JOVE.py**) to automate this process.

271

272　3.2. Quantify fluorescence expression in individual inclusions

273

274　NOTE: To quantify the expression kinetics of the two reporters use the open source image
275　analysis application ImageJ/FIJI and the plugin Trackmate[15]. Trackmate identifies 'spots'
276　(corresponding to inclusions in this case) and follows them through a time-lapse image stack
277　recording the X,Y location and signal intensity for each inclusion over time
278　(**JoVE61365_screenfile14**). This information is saved as a CSV file and will be imported into a
279　custom Python notebook for analysis.

280

281　3.2.1. Open the in-focus Z-reduced image stack in ImageJ/FIJI using Bio-formats Importer by
282　clicking **Plugins | Bio-Formats | Bio-formats Importer**. Select **Hyperstack** and **Composite**
283　(**JoVE61365_screenfile15, JoVE61365_screenfile16**).

284

285　3.2.2. Subtract the image background by clicking on **Process | Subtract Background** using a
286　Rolling ball radius of 50.0 pixels and enhance the image contrast to 0.3% for Saturated pixels
287　(**Process | Enhance Contrast**). Multiply the image values by 10.0 (**Process | Math | Multiply**)
288　(**JoVE61365_screenfile17 - JoVE61365_screenfile22**).

289

290　3.2.3. In Trackmate (**Plugins | Tracking | Trackmate**), select an estimated blob diameter of 48
291　pixels (empirically determined based on the size of the inclusion at the end of imaging). Produce
292　non-fragmented inclusion tracks by selecting a **Linking max distance** and **Gap-closing max**
293　**distance** of 8.0 pixels and **Gap-closing max frame gap** of 1 (**JoVE61365_screenfile23 -**
294　**JoVE61365_screenfile25**).

295

296　NOTE: To improve Trackmate's ability to identify and track inclusions over the entire cycle a
297　separate image channel is created by adding the *euo*prom and *hctB*prom channels together using
298　the image math function in ImageJ/FIJI. This channel is then used by Trackmate to identify and
299　follow inclusions over time. The fluorescent values of the *euo*prom and *hctB*prom channels are
300　then recorded in channels 2 and 3.

301

302　3.2.4. Record tracks that meet a minimum continuous duration: Duration of track: 20
303　(**JoVE61365_screenfile26**). Analyze the tracks and save the **Spots in track statistics** as a CSV file
304　(**JoVE61365_screenfile27**).

305

306 NOTE: This process has been automated using a custom Python script that is provided in the
307 supplemental data (**TrackMate_Zreduced_JOVE.py**).

309 <mark>3.3. Identify inclusion tracks with altered developmental profiles</mark>

311 NOTE: To identify inclusions containing *Chlamydia* with altered developmental profiles, each
312 inclusion track was visualized using Python notebook. These visualizations allow for the
313 identification of inclusions with kinetic gene expression profiles that differed from the mock-
314 treated population. The Python notebook used for identification of inclusions with altered
315 developmental programming is provided in the supplemental data (**EMS_Screen-Markdown**).

317 3.3.1. <mark>Import the inclusion track data from the **Spots in tracks statistics** CSV files into Pandas data
318 frame using the **Import** cell in the EMS_Screen-Markdown Python Notebook.</mark>

320 3.3.2. Baseline correct each track by subtracting the minimum value of each track from the rest
321 of the track values using the **Baseline Subtract** cells. Save the resulting values as a pickle file using
322 the **Save as Pickle** cell. This will permanently save the channel values after baseline subtraction
323 for later retrieval.

325 NOTE: Baseline subtraction sets the starting fluorescent intensity of every inclusion to zero.

327 3.3.3. Eliminate traces from inclusions near the edges of the FOV using the **Filter Edges** cell as
328 their fluorescent profiles may not be fully captured; these traces may produce false positive
329 developmental profiles.

331 3.3.4. Calibrate the frame (**totalFrames**) values from the image slices to time (**startTime, interval**)
332 values with the **Time-lapse Calibration** cell using the experimental start time and imaging time
333 interval. Exclude partial inclusion reads by filtering out traces that do not extend over the last 20
334 h of the experiment (16-36 HPI) using the **Track Duration Filter** cell.

336 3.3.5. Separate tracks into individual data frames by experimental condition with the **Assign
337 Treatment** cell. Filter for inclusions that exhibit sufficient growth using the **Filter for Growth** cell.
338 In the **Filter for Growth** cell, empirically set the fluorescence intensity threshold for the *euo*prom
339 channel by changing the values within the last two lines of code. This will filter out *Chlamydia*
340 that did not grow.

342 NOTE: Be cautious when setting threshold filters, if the filter is too low the resulting scatter plots
343 will be noisy, yet if filtering is set too high important mutants may be eliminated.

345 3.3.6. Calculate the percent mortality caused by EMS by dividing the number of mutagenized
346 tracks/well by the number of mock-treated tracks/well. Multiply the number of mock-treated
347 tracks by the initial dilution factor of 3 to calculate the number of mock-treated tracks/well. Use
348 the **Count Inclusion Tracks** and **Calculate Percent Mortality** cells to perform this task.

350 3.3.7. Calculate the time to half-maximal expression for both early and late reporters for each
351 track using the **Calculate** cell. These values will be used to compare mutagenized and mock
352 populations to identify developmental mutants.
353
354 3.3.8. Within the **Half-Max Plot** cell use the bokeh plotting package to visualize the time to half-
355 max expression of each promoter, graphing the *euo*prom time to half-maximal expression against
356 that of *hctB*prom. Identify inclusions from the mutant population that fall outside the mock-
357 treated scatter cloud using the bokeh interactive track ID explorer. Make note of the FOV and XY
358 coordinates of the inclusions of interest (**Figure 2**).
359
360 NOTE: Pick candidate inclusions that visually fall outside of the control cloud as verification and
361 statistical evaluation of each clone will be performed subsequently.
362
363 3.3.9. Within the **Animated Plot** cell visualize changes in promoter expression kinetics
364 dynamically through time by graphing the expression intensities of *euo*prom against *hctB*prom
365 using the plotting tool Plotly[16]. The scatter function of Plotly is used to animate the gene
366 expression over time (**Video 1**).
367
368 3.3.10. Visualize a snapshot from the Plotly animated graph in the **Inclusion Locator** cell, plotting
369 *euo*prom and *hctB*prom expression at a specific time point (i.e. 28 HPI) using the bokeh package
370 (**Figure 3**). Identify inclusions from the mutant population as described in step 3.3.8.
371
372 NOTE: This analysis needs to be performed quickly (<4 h) as the inclusions are still expanding and
373 will start to lyse host cells ~48 h after infection.
374
375 3.4. Isolate developmental mutants from inclusions of interest
376
377 NOTE: To isolate *Chlamydia* from the inclusions that were determined to display altered gene
378 regulation a micromanipulator with capillary needles was employed. The Well ID, FOV and X,Y
379 coordinates of inclusions of interest were determined using the data visualization in section 3.3.
380
381 3.4.1. Prepare capillary needles by holding the center of the capillary tube in a flame and pulling
382 both ends of the capillary tube until it has separated. Create an opening in the pulled capillary
383 needle by breaking the pulled tip on a microscope slide. To break the needle, place the closed tip
384 on the frosted portion of the microscope slide at an angle and apply pressure.
385
386 NOTE: Capillary tube specifications were 1.0 mm O.D., 0.5 mm I.D.
387
388 3.4.2. Check that the needle opening is approximately the size of an inclusion under a microscope,
389 20x objective.
390
391 3.4.3. Prepull ~25-30 capillary needles for isolation of candidate inclusions (one needle per
392 inclusion).
393

394   3.4.4. Fill the microinjector with mineral oil ensuring that no air bubbles are present.

395

396   3.4.5. Attach a glass capillary needle to the microinjector and expel oil to the tip of the needle,
397   expunging any air bubbles. Place the capillary needle in complete media and draw media up
398   halfway. Filling the capillary needle with media prevents oil contamination in the well.

399

400   3.4.6. Using the saved position list in μManager from step 2.3.5, migrate to the well and FOV of
401   an inclusion of interest identified in the Python visualization notebook.

402

403   3.4.7. Use the joystick of the micromanipulator to localize the capillary needle to the XY
404   coordinates of the inclusion of interest.

405

406   3.4.8. Use the 595 nm excitation channel to visualize EBs for extraction and the phase/DIC white
407   light channel for needle visualization. Maneuver the capillary needle to the inclusion, rupture the
408   inclusion and then draw the EBs into the capillary needle using the microinjector.

409

410   3.4.9. Expel the EBs from the capillary needle into a single well of the prepared 24 well
411   polystyrene plate prepared in step 2.1.2. Remove the capillary needle and replace with a fresh
412   capillary needle for next inclusion extraction. Repeat section 3.4 for all candidate inclusions.

413

414   NOTE: For expansion and to ensure a high enough titer for re-imaging, incubate mutant isolates
415   in a 5% CO_2, 37 ˚C incubator until the majority of the host cells are infected (~1 week). Wells
416   should be monitored closely as different isolates may exhibit different growth rates.

417

418   3.5. Harvest mutant isolates

419

420   3.5.1. On ice, disrupt the infected monolayer by scraping with a 1 mL micropipette tip. Transfer
421   the media, cell debris, and released *Chlamydia* into a 1.5 mL microcentrifuge tube.

422

423   3.5.2. Pellet *Chlamydia* by centrifugation for 30 min at 4 ˚C, >14,000 *x g*. Remove the
424   supernatant and resuspend pellet in 75 μL of ice cold 1x SPG. Aliquot into three 1.5 mL screw-
425   cap microcentrifuge tubes. Store at -80 ˚C.

426

427   **4. Verification of mutant isolate phenotypes**

428

429   4.1. Host cell culture for imaging mutagenized isolates

430

431   4.1.1. Seed a 96 well glass bottom plate with 1.6 x 10^4 Cos-7 cells (ATCC) per well in 100 μL of
432   complete media. Incubate at 5% CO_2, 37 °C. Cells should reach confluency in approximately 24 h.
433   After cells are confluent, replace media with complete media supplemented with 1 μg/mL
434   cycloheximide, incubate overnight.

435

436   4.2. Infect cells with candidate isolates for phenotypic verification

437

438   4.2.1. Thaw mutant clones and wildtype *Chlamydia* on ice.

439

440   4.2.2. In the prepared 96 well plate, perform a two-fold serial dilution of mutant isolates, using
441   one column per isolate (11 columns). Start with an initial dilution of 1:20 in 100 µl HBSS.

442

443   NOTE: Serial dilution of *Chlamydia* is performed to ensure mutant samples are imaged at an MOI
444   < 1.

445

446   4.2.3. Infect the remaining (12th) column with wildtype *Chlamydia* at MOI ~0.5.

447

448   NOTE: Wildtype *Chlamydia* are used as a control for comparison against mutagenized isolates.

449

450   4.2.4. Incubate for 15 min rocking at 37 °C.

451

452   4.2.5. Wash infected host cells with prewarmed (37 °C) HBSS with 1 mg/mL of heparin and HBSS
453   as specified in section 2.2.

454

455   4.2.6. Replace with 200 µL per well of prewarmed (37 °C) imaging media.

456

457   4.2.7. Fill the interwell spaces with prewarmed (37 °C) deionized H_2O.

458

459   4.2.8. Incubate at 5% CO_2, 37 °C for 10 h.

460

461   4.3. Microscope setup

462

463   NOTE: Refer to section 2.3 for microscope setup, this section will only contain the required setup
464   modifications.

465

466   4.3.1. Select the **96 well plate template** from the **HCS** plugin.

467

468   4.3.2. Empirically determine wells corresponding to an MOI < 1 for each mutant isolate. Clover
469   expression under control of the *euo* promoter is observable at ~10 HPI making early
470   visualization of inclusions possible (**Figure 1B**).

471

472   4.3.3. Select three wells per mutant isolate that correspond to an MOI < 1 and generate an
473   imaging position list consisting of two FOV per well.

474

475   NOTE: Only 72 images can be taken per time interval due to hardware constraints, this equates
476   to three dilutions (wells) per strain using two imaging sites per well if 12 samples are imaged.

477

478   4.3.4. Record the developmental cycle of each mutant isolate for 36 h at 30 min time intervals
479   starting at 12 HPI.

480

481 4.3.5. Record the experimental details in the **Acquisitions Comments** box. i. e., Well ABC1:
482 wildtype control. Well ABC2: Mutant strain 1, ABC3: Mutant strain 2, etc… Imaging: 12-48 HPI.
483
484 4.3.6. Start the image acquisition at 12 HPI.
485
486 **5. Data analysis for isolate verification**
487
488 5.1. Create in-focus image stacks and quantify fluorescence expression in individual inclusions
489
490 5.1.1. Generate fluorescent intensity traces for each inclusion as specified in section 3.1 - 3.2.
491
492 5.2. Verify Ctr mutagenized isolates
493
494 NOTE: To verify the altered developmental profiles of mutant isolates, their expression profiles
495 are compared to the wildtype expression profile using Python notebook. The Python notebook
496 used for verification of mutant clones with altered developmental programming is provided in
497 the supplemental data (**clone_check-Markdown**).
498
499 5.2.1. Import and filter the inclusion trace data in the clone_check-Markdown Python notebook
500 as done in section 3.3.
501
502 5.2.2. Calculate the mean and standard deviation (STD) from the traces of each isolate and
503 wildtype control population using the **Calculate Mean & STD** cell.
504
505 5.2.3. With the **Graph Iso vs WT** cell plot the mean and standard error of the mean (SEM) of each
506 mutant clone against the wildtype control to determine if the mutant expression kinetics are
507 divergent from the wildtype sample (**Figure 4**).
508
509 5.2.4. Determine if the isolated mutant population is clonal by plotting the mutant traces and
510 comparing them to wildtype inclusion traces using a scatter plot as done in section 3.3 (steps
511 3.3.7-3.3.10) (**Figure 2,3**). If the isolate is a mixed population the plot will show one population
512 overlaying with wildtype and a second distinct population outside of the wildtype scatter cloud.
513 If the population looks mixed the mutant can be re-isolated using the original procedure
514 described in section 3.4.
515
516 NOTE: To determine if the developmental profile of an isolate is statistically different from
517 wildtype the curves for each isolate should be compared to wildtype using ANOVA.
518
519 **REPRESENTATIVE RESULTS:**
520
521 Direct EMS mutagenesis of our promoter-reporter chlamydial strain resulted in an ~75%
522 reduction in infectivity. Using the described live-cell imaging protocol, ~600 inclusions imaged
523 and tracked over a 24 h period. The fluorescent expression kinetics of both reporters in each
524 inclusion was visualized using custom Python notebook scripts. Two visualization approaches

525 were implemented to identify candidate mutagenized *Chlamydia* for isolation. The first
526 methodology (step 3.3.8) visualizes the time to half-maximal expression of *euo* and *hctB*
527 promoters from individual chlamydial isolates in an interactive scatter plot (**Figure 2**). Inclusions
528 were identified for isolation if they fell outside the mock-treated scatter cloud. Candidate clones
529 were picked that visually fell outside of the control cloud. Verification of each clone was
530 performed subsequently. Clones A3-6-67 and B3-8-58 were selected for isolation as they
531 produced shorter times to half-maximal expression from the *euo* promoter and longer times for
532 *hctB* (**Figure 2**).
533
534 The second visualization method for identifying inclusions with altered kinetics (steps 3.3.9-10)
535 identifies individual inclusions based on visualization of dynamic gene expression from the two
536 promoters (**Video 1**). Again, candidate clones with dynamic inclusion expression patterns that
537 were noticeably distinct from control inclusions were picked. B3-6-62 was chosen due to
538 increased fluorescent accumulation from the *euo* promoter between 23 and 29 HPI (**Video 1**). A
539 snapshot of the animated graph was taken to identify the location of the inclusions of interest
540 (**Figure 3**).
541
542 Using the two visualization methods, a total of 24 inclusions were identified for isolation. Of the
543 24 total isolates, 10 showed differential kinetics upon retesting. These isolates fell into three
544 phenotypic categories; 8 isolates exhibited decreased *euo*prom expression at ~24 HPI,
545 corresponding to the time of RB-EB conversion, as demonstrated by the clone A3-6-67 (**Figure
546 4A**). The remaining two clones displayed unique phenotypic profiles, the B3-8-58 isolate also
547 exhibited decreased *euo*prom expression at ~24 HPI, yet an overall increase in *hctB*prom
548 expression (**Figure 4B**), whereas B3-6-62 expressed increased levels of fluorescence from the *euo*
549 promoter followed by a sudden loss of expression in both promoters (**Figure 4C**). Analysis of the
550 live-cell micrographs for mutant B3-6-62 revealed that host cell lysis occurred in cells infected
551 with this mutant much earlier than in wildtype infected cells (**Video 2**).
552
553 **FIGURE AND TABLE LEGENDS**
554 **Figure 1: Monitoring cell-type development with Ctr promoter-reporters.** (**A**) Schematic of the
555 promoter-reporter construct, p2TK2-*hctB*prom-mKate2/*euo*prom-Clover. (**B**) Live-cell
556 micrograph of *euo*prom-Clover and *hctB*prom-mKate2 expression in Ctr at 10 HPI (**C**) Live-cell
557 micrograph of Ctr expressing *euo*prom-Clover and *hctB*prom-mKate2 at 36 HPI. Scale bar: 20 μm.
558
559 **Figure 2: Identification of representative isolates A3-6-67 and B3-8-58 by visualization of the
560 time to half-maximal expression for each promoter.** The interactive graph is used to identify
561 mutagenized *Chlamydia* exhibiting expression profiles that differ from the mock-treated control
562 scatter cloud. Each spot on the graph represents a single inclusion. Inclusion spots A3-6-67 and
563 B3-8-58 are highlighted as they fall outside of the mock-treated cloud, both exhibiting shorter
564 time to half-maximal expression of the *euo* promoter in combination with longer time to half-
565 maximal expression of *hctB*. *euo*prom: x-axis, *hctB*prom: y-axis.
566
567 **Video 1: Identification of mutagenized *Chlamydia* exhibiting divergent expression kinetics
568 using a dynamic gene expression plot.** Promoter expression of *euo* and *hctB* for individual

569   inclusions were plotted and visualized through time to identify mutagenized *Chlamydia* with
570   altered expression dynamics. B3-6-62 was chosen for isolation as it exhibits higher *euo*prom
571   expression in comparison to the wildtype cloud. *euo*prom: x-axis, *hctB*prom: y-axis.

573   **Figure 3: Interactive snapshot for identification of inclusion location.** The graph presented is a
574   snapshot at 28 HPI from the animated scatter plot (**Video 1**) and was used to identify the FOV
575   and XY coordinate location of inclusions of interest. B3-6-62 is shown as it was chosen for
576   isolation from the animated scatter plot.

578   **Figure 4: Verification of representative mutant isolates.** Developmental profiles of mutagenized
579   isolates A3-6, B3-8, and B3-6. (**A**) The A3-6 mutant exhibits a decrease *euo*prom expression at
580   ~24 HPI. (**B**) The B3-8 mutant isolate exhibits a decrease *euo*prom expression at ~24 HPI, but an
581   overall increase in *hctB*prom expression. (**C**) The B3-6 isolate exhibits increased levels of *euo*prom
582   expression followed by a sudden loss of expression in both promoters at ~40 HPI. Each sample is
583   the average of the specified population, n > 25. Cloud represents SEM.

585   **Video 2: Representative mutant B3-6 causes premature host-cell lysis.** Time-lapse live-cell
586   micrograph of B3-6 infected host-cells undergo premature lysis (~40 HPI).

588   **Figure 5: Workflow for directed forward genetic analysis of promoter-reporter Ctr:** Ctr-L2-
589   p2TK2-*hctB*prom-mKate2/*euo*prom-Clover EBs were directly mutagenized with EMS in axenic
590   media, CIP-1. Mutagenized EBs were used to infect Cos-7 cell monolayers for imaging and
591   fluorescent expression analysis. *Chlamydia* expressing altered developmental dynamics were
592   identified by visualization in interactive graphs. Inclusions with altered developmental profiles
593   were isolated using a micromanipulator. The phenotypes of the isolates were verified upon
594   reinfection. Mutant isolates are subjected to WGS to identify DNA lesions associated with
595   phenotypes.

597   **DISCUSSION:**
598   Dissecting the mechanisms that control the chlamydial developmental cycle has been hindered
599   by the limitations of the currently available genetic tools. Employing our promoter-reporter
600   *Chlamydia* in conjunction with live-cell automated microscopy, a system was built which enables
601   monitoring of cell-type development in individual inclusions over a 24 h period. This system, in
602   combination with chemical mutagenesis and direct inclusion isolation has established a method
603   to rapidly and clonally select *Chlamydia* expressing altered developmental profiles (**Figure 5**).

605   Chlamydial EBs are metabolically active outside the host when provided with intracellular ionic
606   conditions and an energy source[5,12]. This EB axenic metabolism was leveraged to mutagenize
607   purified EBs outside of host cells. In this protocol, metabolizing EBs were directly mutagenized
608   with EMS. It was observed that EMS treatment effectively reduced EB viability and generated EBs
609   that produced variable developmental kinetics as expected.

611   It's estimated that the described EMS mutagenesis protocol generates ~5-20 DNA changes/EB.
612   The live-cell microscopy workflow described is capable of imaging ~8 inclusions per field of view

613 (FOV) and 72 FOVs every in a 30 min interval. Therefore, it is estimated that the effects of ~3000-
614 10,000 mutations can be visualized per run. Multiple runs (3-5) will result in visualization of the
615 effects of 9,000-50,000 mutations. The Ctr-L2 genome encodes ~850 genes, suggesting this
616 protocol will result in the visualization of >10 mutations per gene. These estimates indicate that
617 genome coverage, while not complete, should be sufficient.
618
619 The strength of this protocol is the ability to track and record the expression kinetics of multiple
620 promoter reporters at the single inclusion resolution in near real-time. Forward genetics relies
621 on observable phenotypes and clonal isolation. Past methods for forward genetics in *Chlamydia*
622 relied on static observations and plaquing with agar overlays[8]. With our methodology, dynamic
623 promoter activity is recorded throughout the developmental cycle and then visualized to identify
624 inclusions that contain *Chlamydia* with altered gene expression kinetics. Identifying candidate
625 inclusions using multiple parameters (i.e., the time to half-maximal expression and total
626 fluorescent intensity at a given time point) results in distinct mutant pools that display different
627 developmental kinetics. These *Chlamydia* are likely to have unique mutations that affect the
628 regulation of separate genetic pathways. The fact that these profiles can be recorded live and
629 visualized after a few hours allows time to locate and isolate the inclusions of interest from the
630 infected monolayer. Although we focused on the gene expression dynamics during development,
631 alternative gene reporters can be used to probe other regulatory pathways.
632
633 Depending on the genetic pathways being interrogated, caution should be taken with the
634 addition of cycloheximide to host cells. Although incubation with cycloheximide improves the
635 imaging characteristics of the monolayer by blocking replication of the host cells; this effect is
636 achieved through inhibiting host protein synthesis. Inhibition of de novo host protein synthesis
637 could influence the results of the genetic screen depending on the question asked.
638
639 Phototoxicity and photobleaching are major hurdles in long-term time-lapse microscopy. To
640 overcome these issues, the specific characteristics of each fluorescent protein should be
641 considered prior to experimentation. Clover and mKate2 have short maturation times (20-30 m)
642 are photostable, and exhibit relatively large quantum yields[17,18]. These qualities allow for the
643 reduction of excitation intensity and exposure time, thus reducing the amount of phototoxicity
644 and photobleaching incurred. The phase/DIC white light channel was employed for autofocusing
645 as this spectrum of light was less phototoxic to *Chlamydia.*
646
647 For this protocol, EMS was used as a chemical mutagen. EMS causes G:C to A:T transitions via
648 guanine alkylation[19]. However, this protocol can be expanded to include alternative mutagens
649 that can induce other kinds of genomic mutations. For instance, acridines are a class of DNA
650 intercalating compounds which induce indels, increasing the chance of frame shifts and therefore
651 null mutations[20].
652
653 With advances in chlamydial transformation techniques, mutated genes that are associated with
654 phenotypic complementation groups can be knocked out via insertional gene disruption and
655 genetic complementation for verification of genotype-phenotype linkage[9]. Recovering mutants
656 that block RB to EB development could be problematic as mutations of interest may produce

657 *Chlamydia* that cannot reinfect host cells. This technique can be modified to identify
658 developmental genes by statistical associations (GWAS). The genomes of *Chlamydia* from
659 isolated inclusions can be directly sequenced without expansion and verification. The high
660 throughput nature of this technique would make statistical associations possible. Again,
661 verification of these associations can be tested through gene disruption and complementation[9].
662

668

669 **DISCLOSURES:**
670 The authors have nothing to disclose.
671

672 **REFERENCES:**
673

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Figure 1

Figure 2

Figure 3
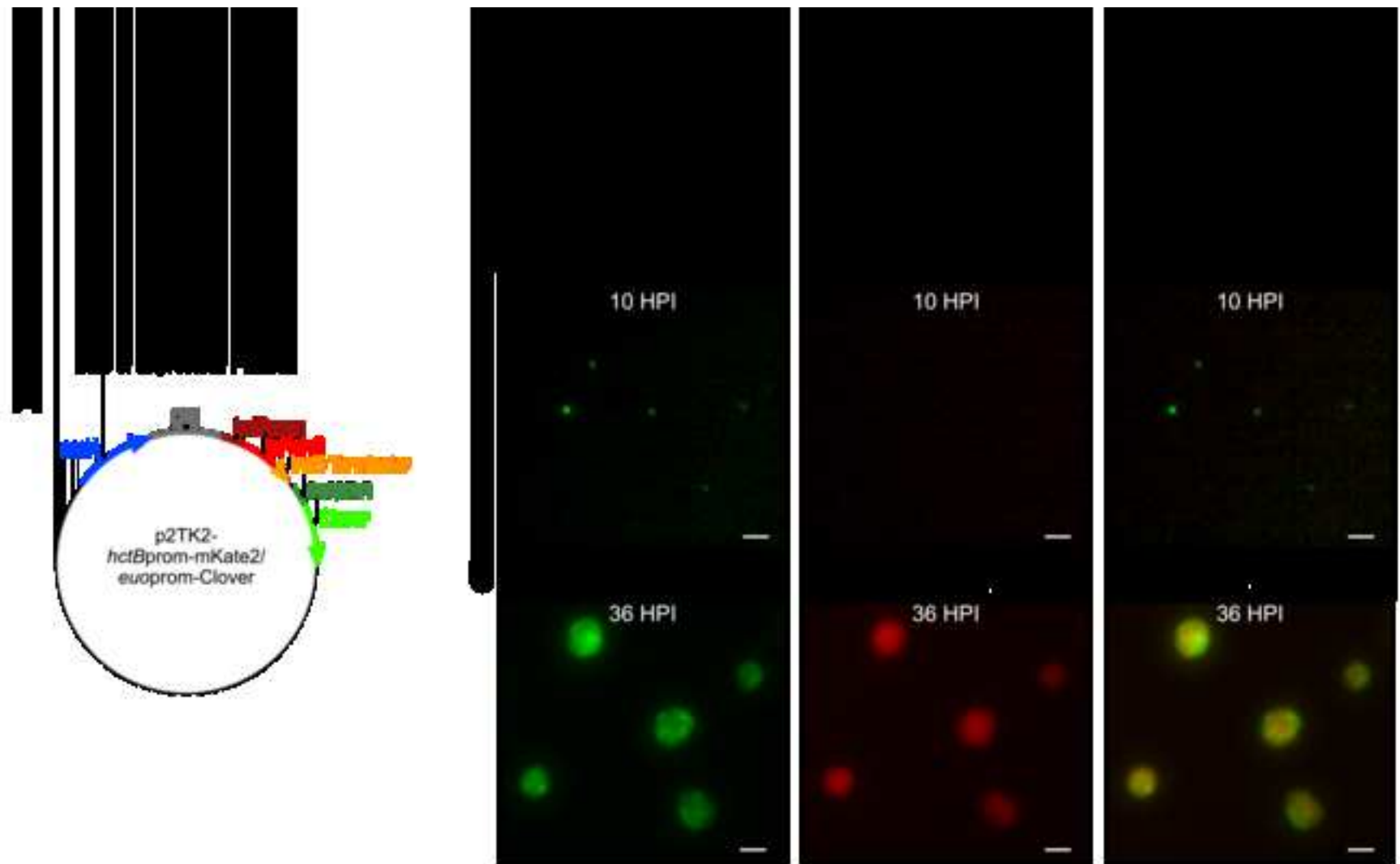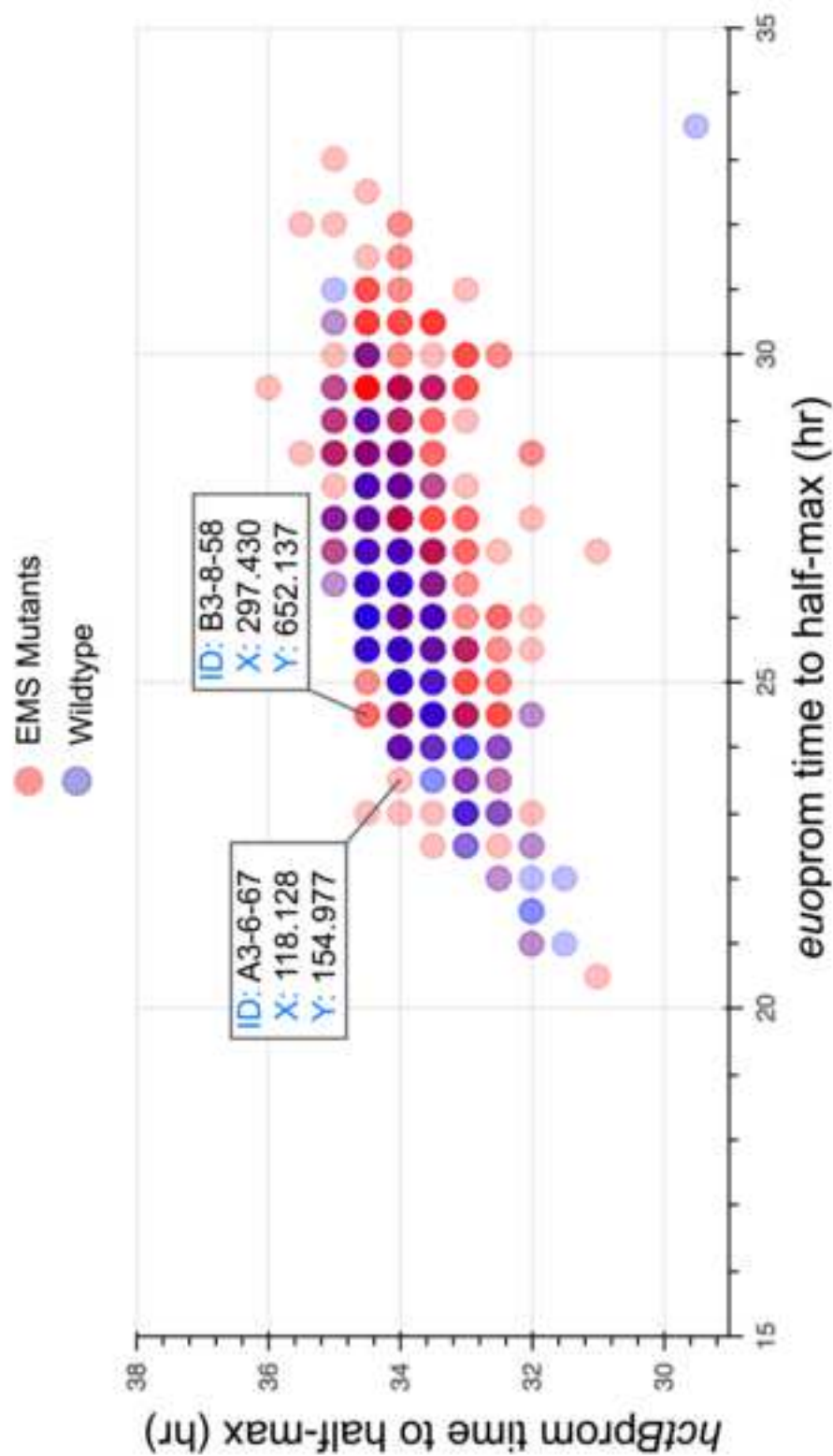
Figure 4
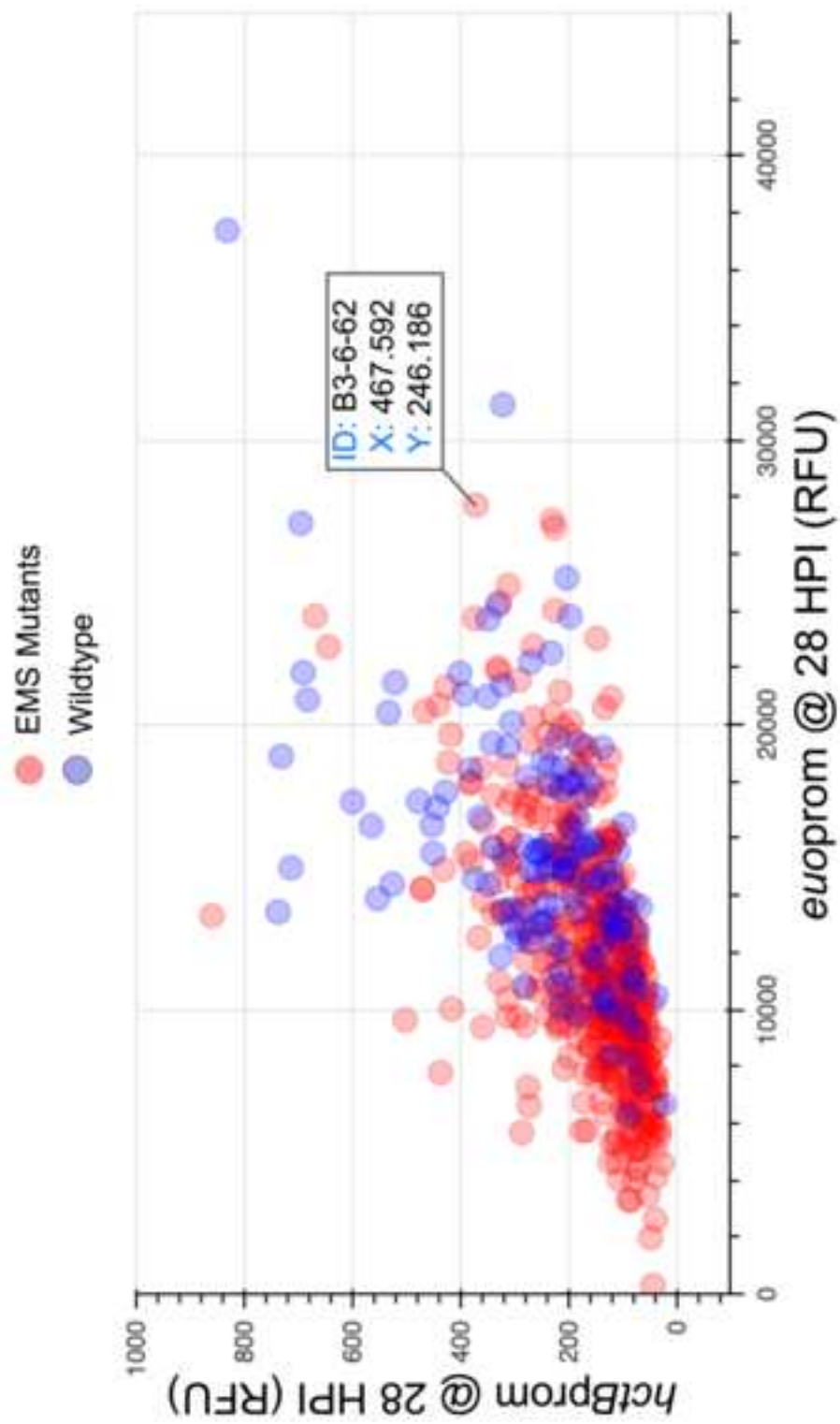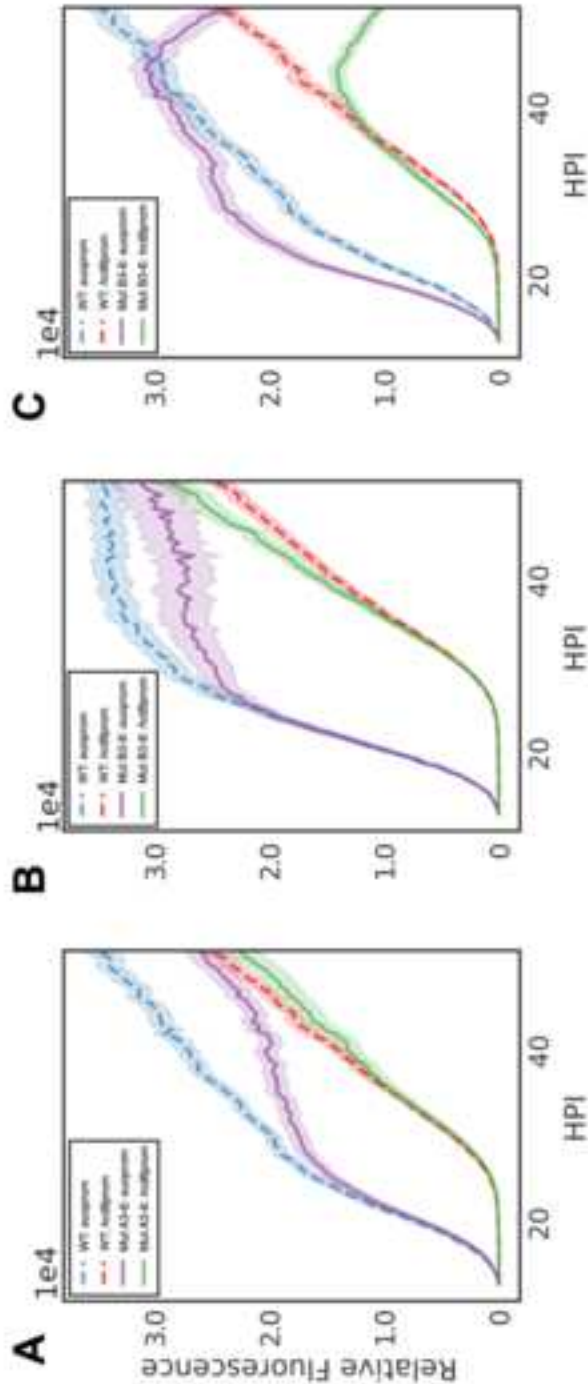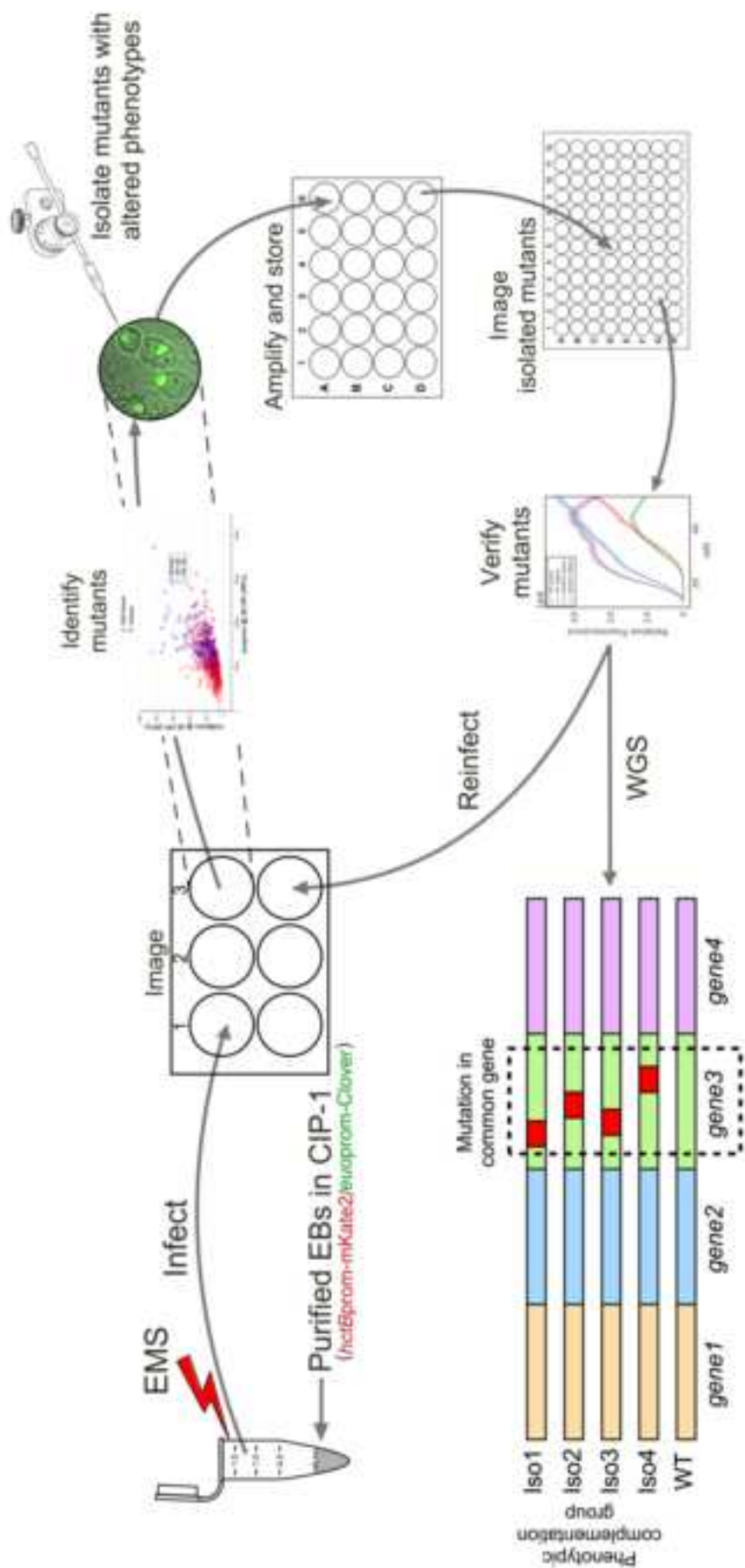
Figure 5

Video_1

Click here to access/download
Video or Animated Figure

Animated_gene_expression_plot.mov

Video_2

Click here to access/download

Video or Animated Figure

B3-6_final.mov

Table of Materials

Name of Material/Equipment
24-well polystyrene plates
6-well glass bottom plates
96-well glass bottom plates
Bold line CO2 Unit
Bold line T Unit
Borosilicate glass capillary tubes
BrightLine bandpass emissions filter (514/30nm)
BrightLine bandpass emissions filter (641/75nm)
CellTram Vario
Chlamydia trachmatis serovar L2
CIP-1 media
Cos-7 cells (ATCC)
Cycloheximide
Ethyl methanesulfonate, 99%
Fetal Plex
Fiji/ImageJ
Galaxy 170 S CO2 incubator
gblocks (Fluorescent FP variants: Clover and mKate2)
Gentamycin 10mg/ml
HBSS (Hank's Balanced Salt Solution)
Heparin sodium
HEPES 1M
InjectMan
Jupyter Notebook
Lambda 10-3
Oko Touch
Prior XY stage
PrismR Centrifuge
Problot Hybridization oven
Proscan II
Purifier Class 2 Biosafety Cabinet
RPMI-1640 (no phenol red)
RPMI-1640 (phenol red)
scopeLED excitation LEDs (470nm,595nm)
Sonic Dismembrator Model 500
Stage incubator
sucrose-phosphate-glutamate buffer 1X (SPG)
T-75 Flasks
TE 300 inverted microscope
THOR LED
Trypsin
Zyla sCMOS
μManager 2.0gamma

Company	Catalog/Part Number
Corning	3524
Cellvis	P06-1.5H-N
Nunc	165305
OKO Labs	CO2 UNIT BL
OKO Labs	H301-T-UNIT-BL-PLUS
Sutter Instrument	B1005010
Semrock	FF01-514/30-25
Semrock	FF02-641/75-25
Eppendorf	5196000030
ATCC	VR-577
In house	NA
ATCC	CRL-1651
MP Biomedicals	194527
Acros Organics	AC205260100
Gemini Bio-Products	100-602
https://imagej.net/Fiji	NA
Eppendorf	CO1700100X
Integrated DNA Technologies	NA
Gibco	15710-064
Corning	21-020-CM
Amersham Life Science	16920
GE Life Sciences	SH30237.01
Eppendorf	5179 000.018
https://jupyter.org/	NA
Sutter Instrument	LB10-3
OKO Labs	Oko Touch
Prior	H107
Labnet	C2500-R
Labnet	H1200A
Prior	H30V4
Labconco	362804
Gibco	11835-030
GE Life Sciences	SH30027.01
scopeLED	F140
Fisher Scientific	15-338-550
OKO Labs	H301-K-FRAME
In house	NA
Thermo Scientific	156499
Nikon	16724
Thor Labs	LEDD1B
Corning	25-052-CI
Andor	ZYLA-5.5-USB3
https://github.com/micro-manager/micro-manager	NA

Comments/Description
Cell culture growth for reinfection of isolates
Cell culture growth for imaging
Cell culture growth for imaging
Stage incubator CO2 control
Stage incubator temperature control
Capillary tubes
Fluoescent filter cube
Fluoescent filter cube
Microinjector
Chlamydia trachomatis
Axenic media. IPB supplemented with 1% FBS, 25 μM amino acids, 0.5mM G6P, 1.0 mM ATP, 0.5 mM DTT
African green monkey kidney cell (host cells)
Host cell growth inhibitor
Mutagen
Supplement for base growth media
Open sourse Image analysis software. https://imagej.net/Fiji
Cell culture incubation
gblock ORFs of Ctr optimized FP varients for cloning into p2TK2SW2
Antibiotic for growth media
Host cells rinse
inhibits and reverses the early electrostatic interactions between the host cell and EBs
pH buffer for growth media
Micromanipulator
Visualization of inclusion traces. https://jupyter.org/
Filter wheel controler
Interface to control the Bold line T and CO2 Unit
Motorized XY microscope stage
Temperature controlled microcentrifuge
Rocking Incubator for infection with Chlamydia
XYZ microscope stage controler
Cell culture work
Base growth media for imaging
Base growth media
Excitation light
Sonicator, resuspending chlamydial pellet
Cluster well plate incubation chamber
Chlamydial storage buffer. (10 mM sodium phosphate [8 mM K 2HPO 4, 2 mM KH 2PO 4], 220 mM sucro
Cell culture growth
microscope
White light
Dislodges host cells from flask for seeding into plates
imaging camera
Open sourse automated microscope control software package

, and 50 µM GTP, UTP, andCTP. (Omsland, A. 2012) made in-house.

se, 0.50 mM L-glutamic acid; pH 7.4)

Response to the editor

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision..

*This has been don*e.

2. Please address all the specific comments marked in the manuscript.

All comments have been addressed.

3. Once done please ensure that the protocol section length is no more than 10 pages and the highlight is no more than 2.75 pages including headings and spacings.

Once you submit, I will put this up in the finalization pipeline.
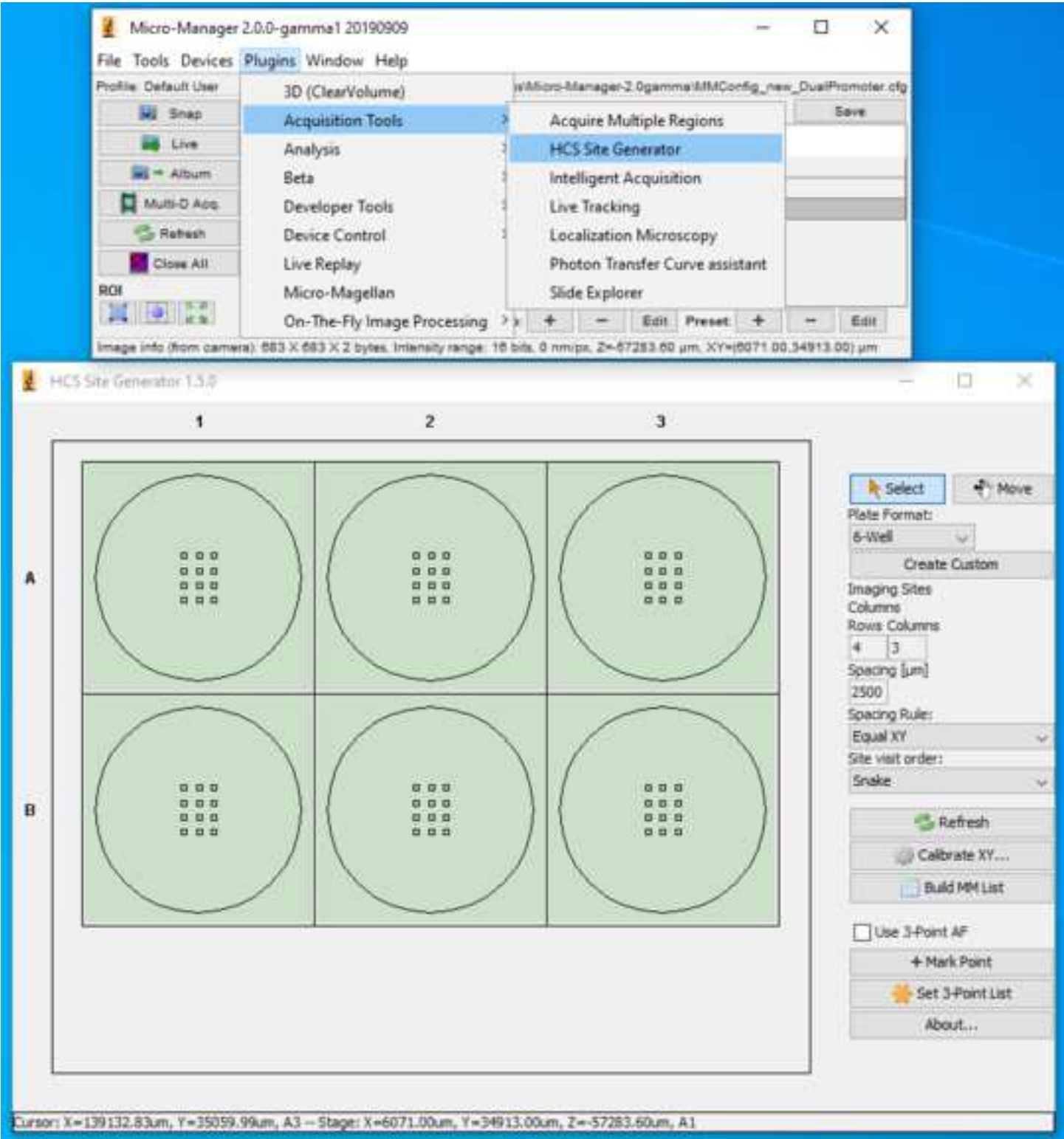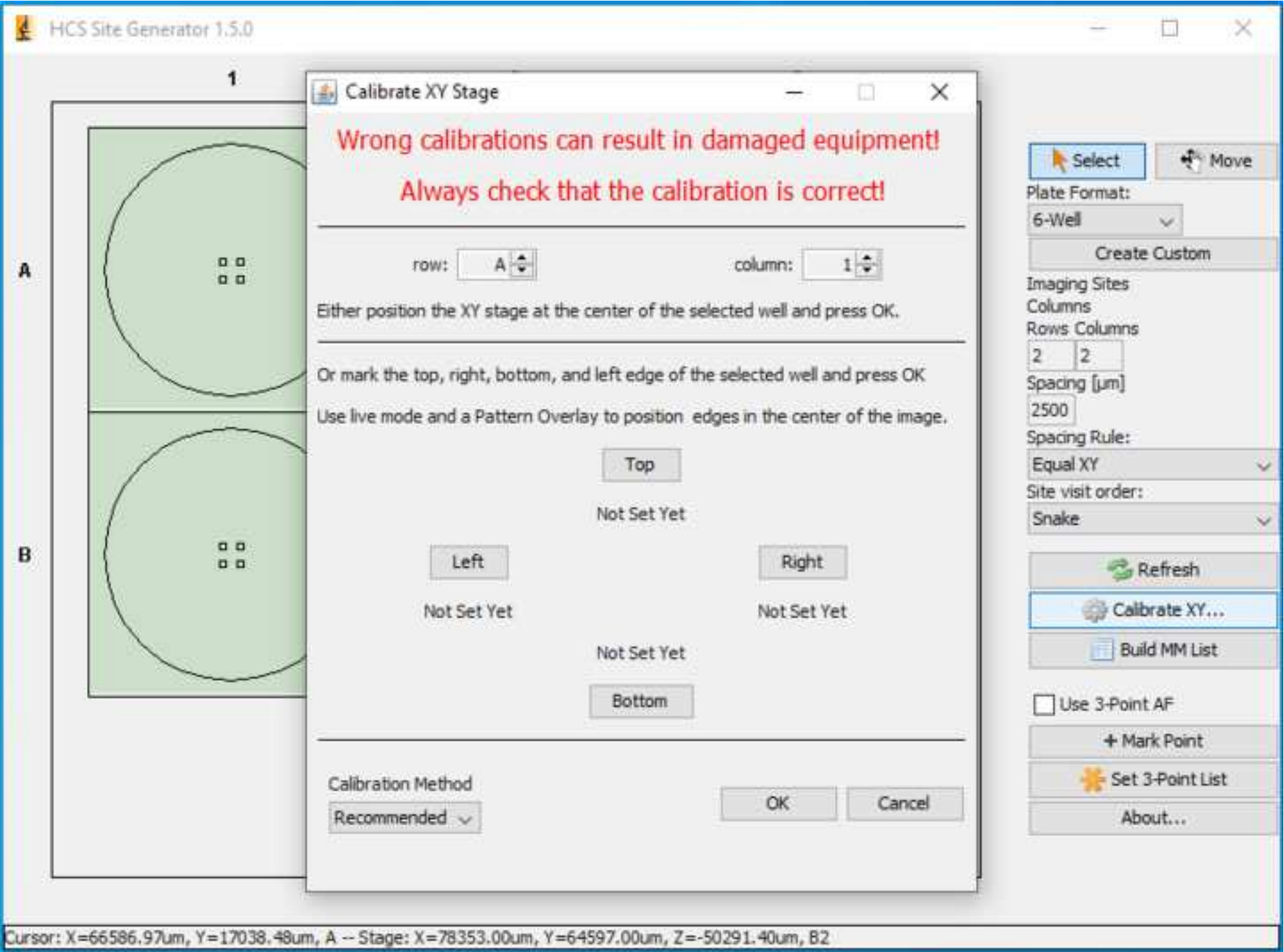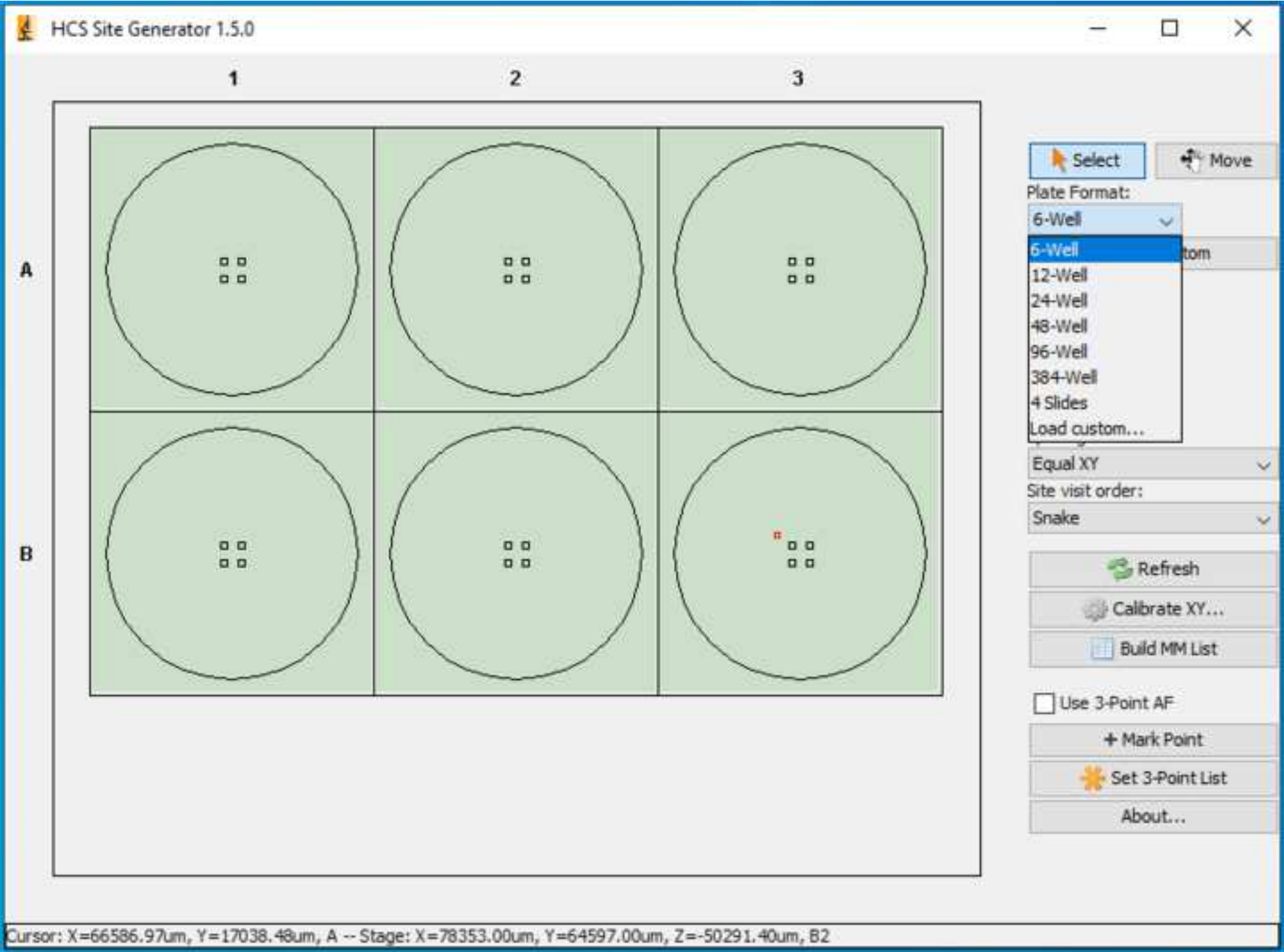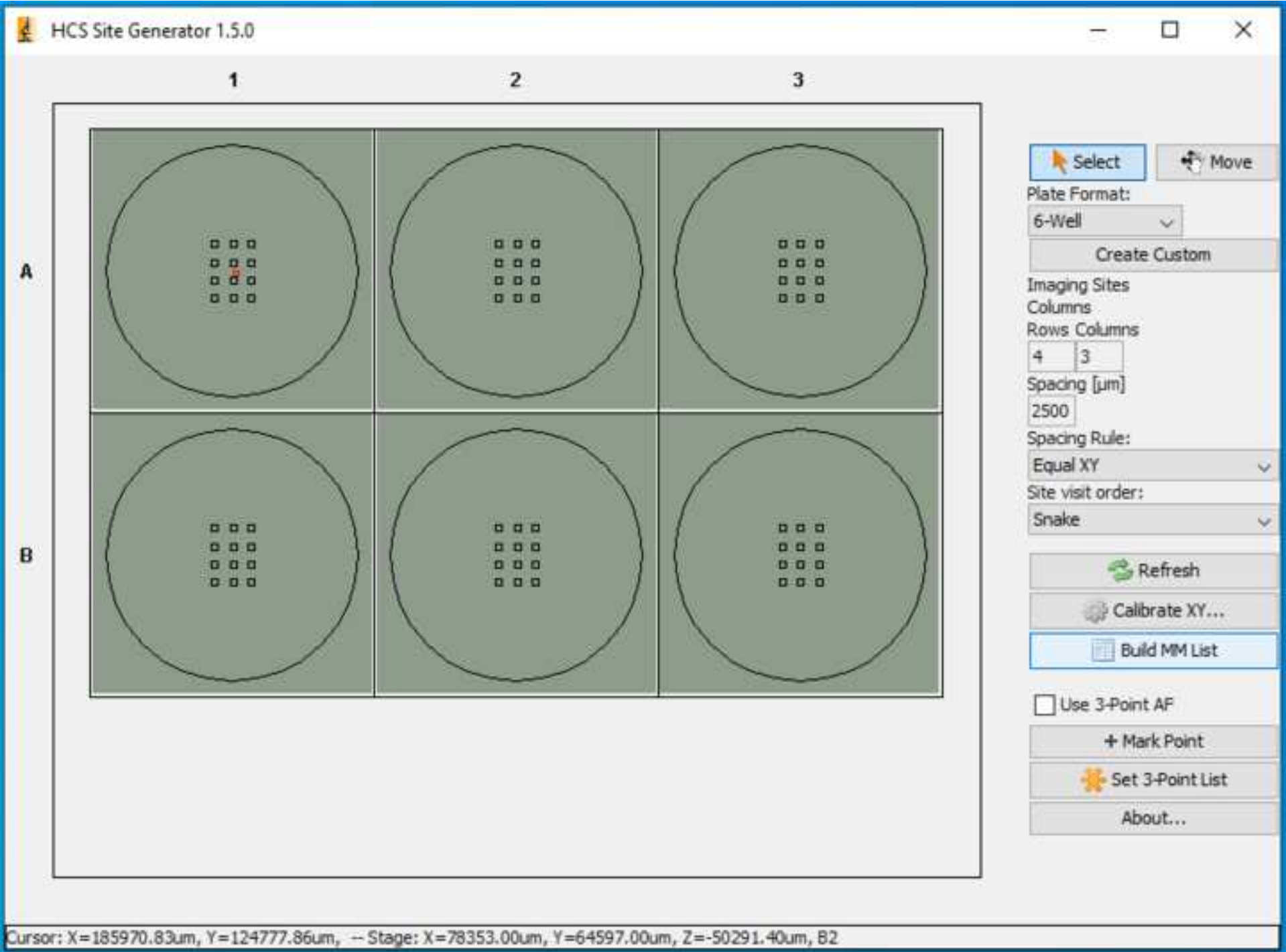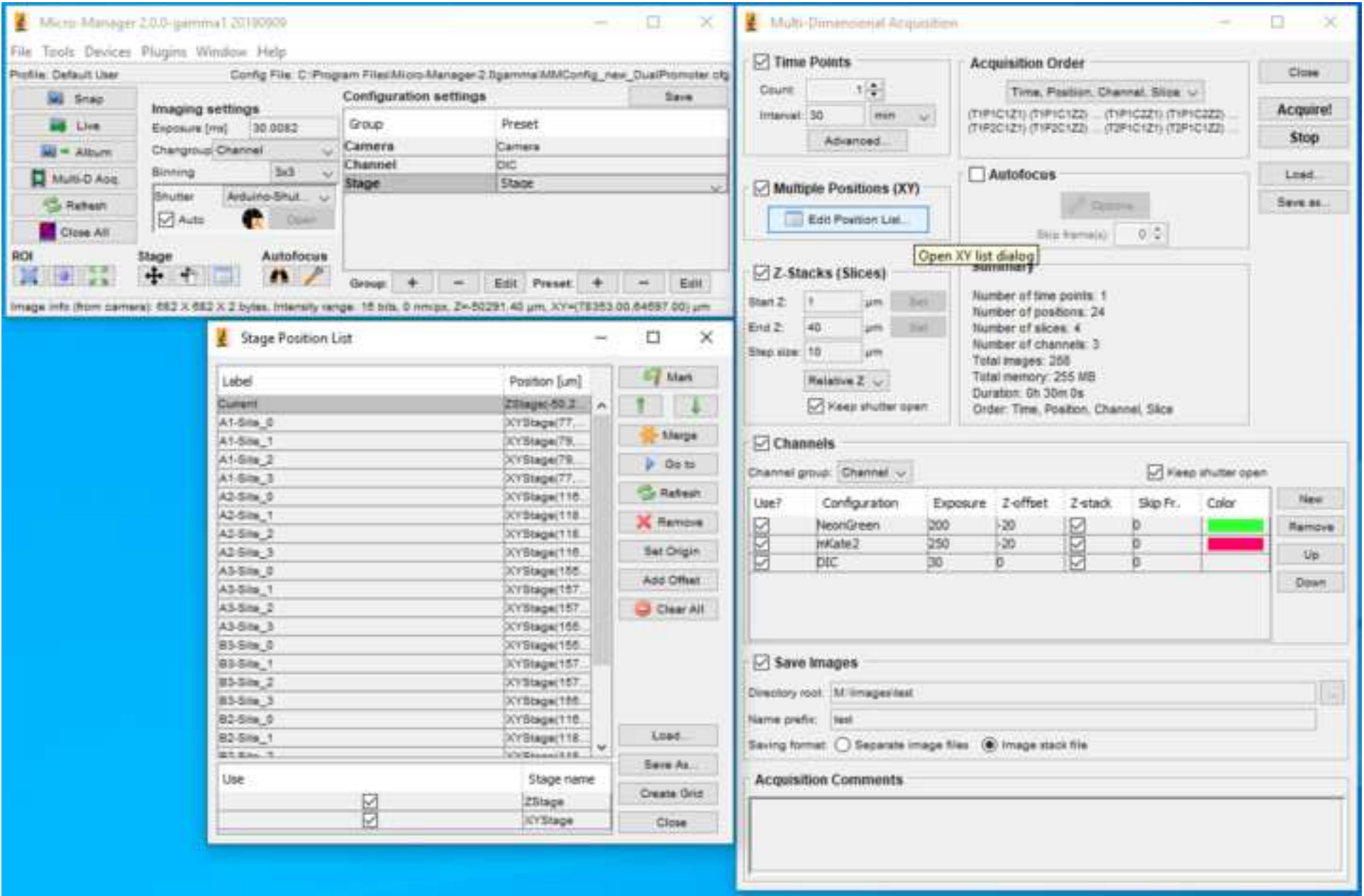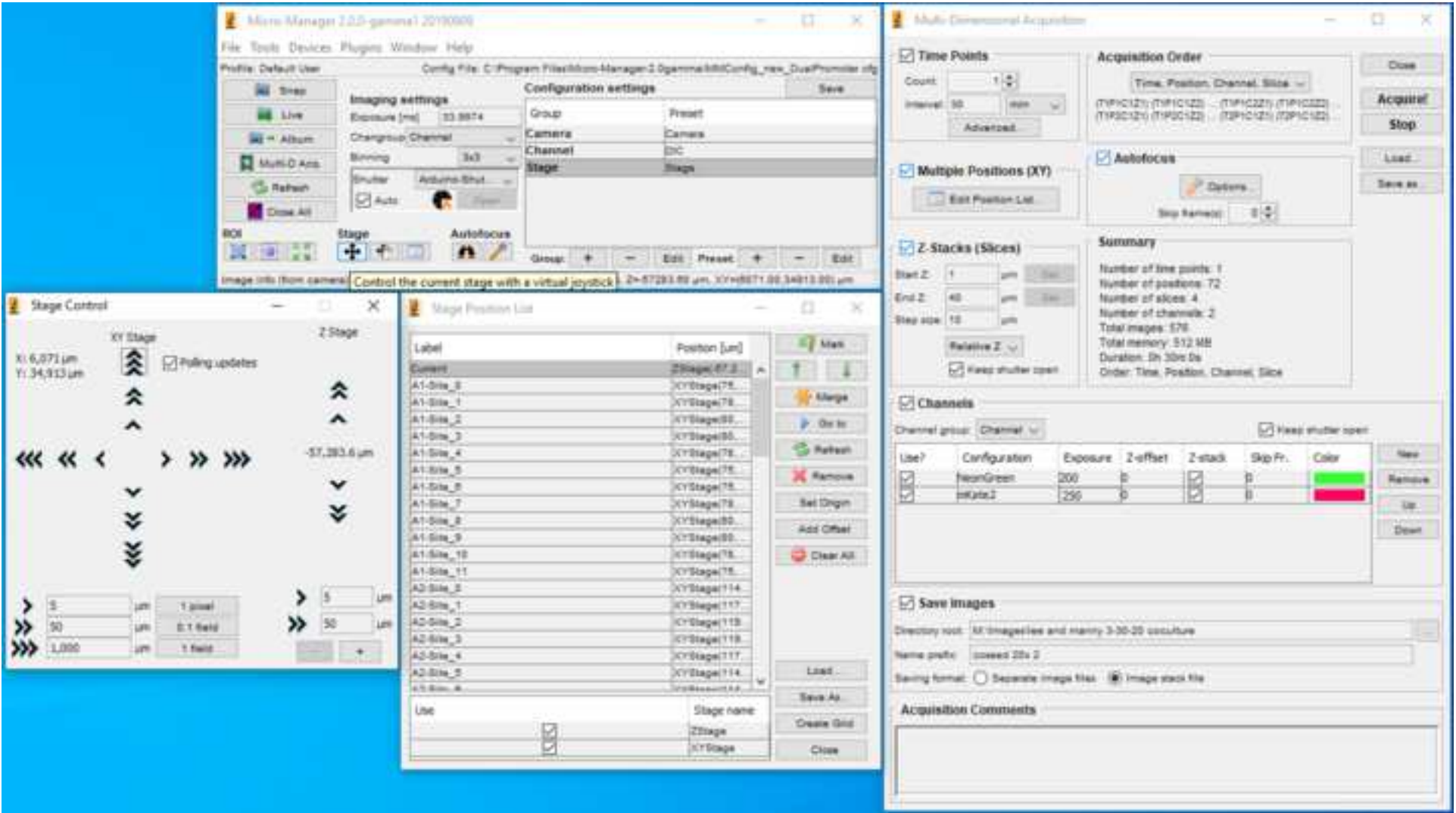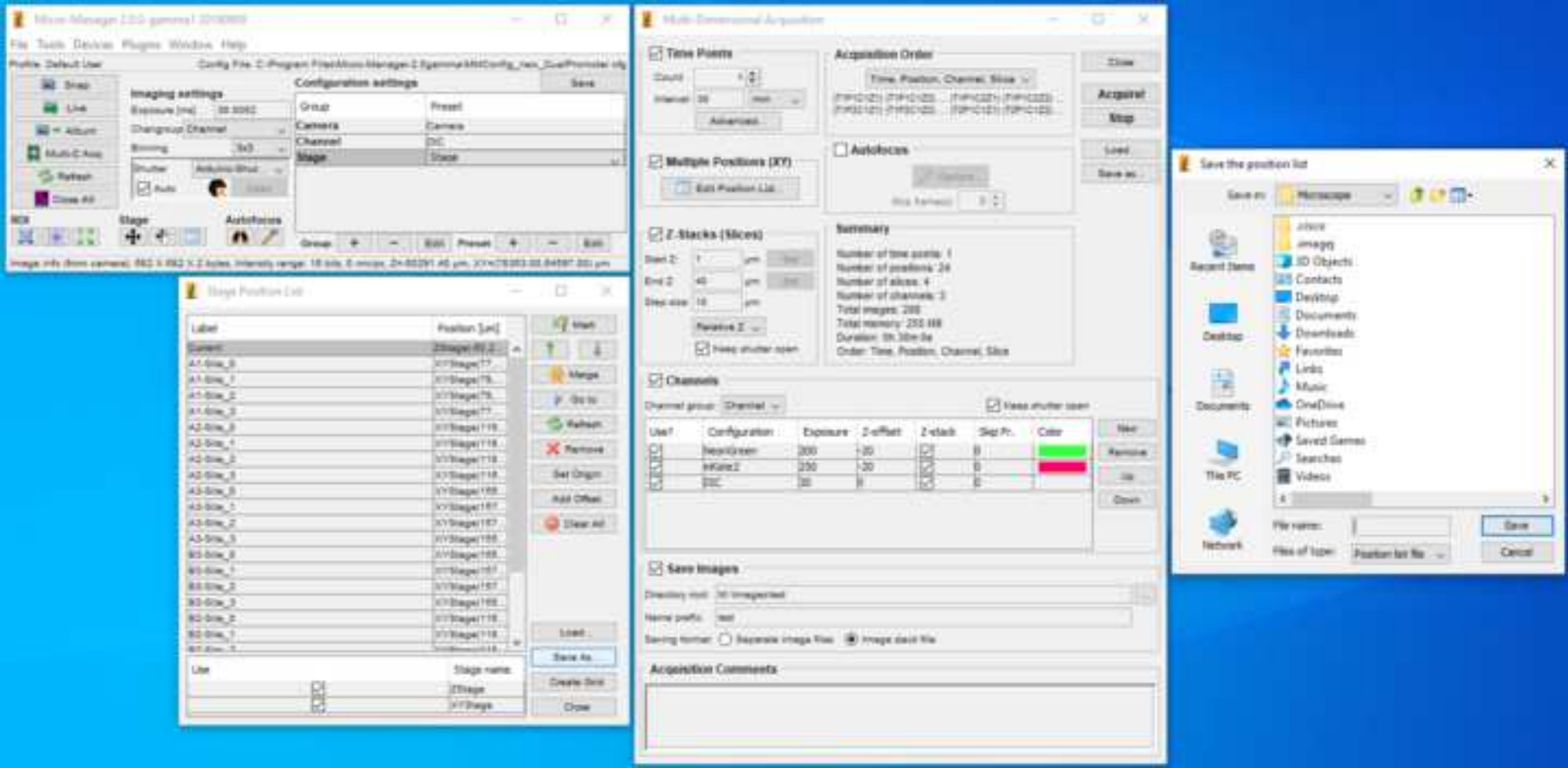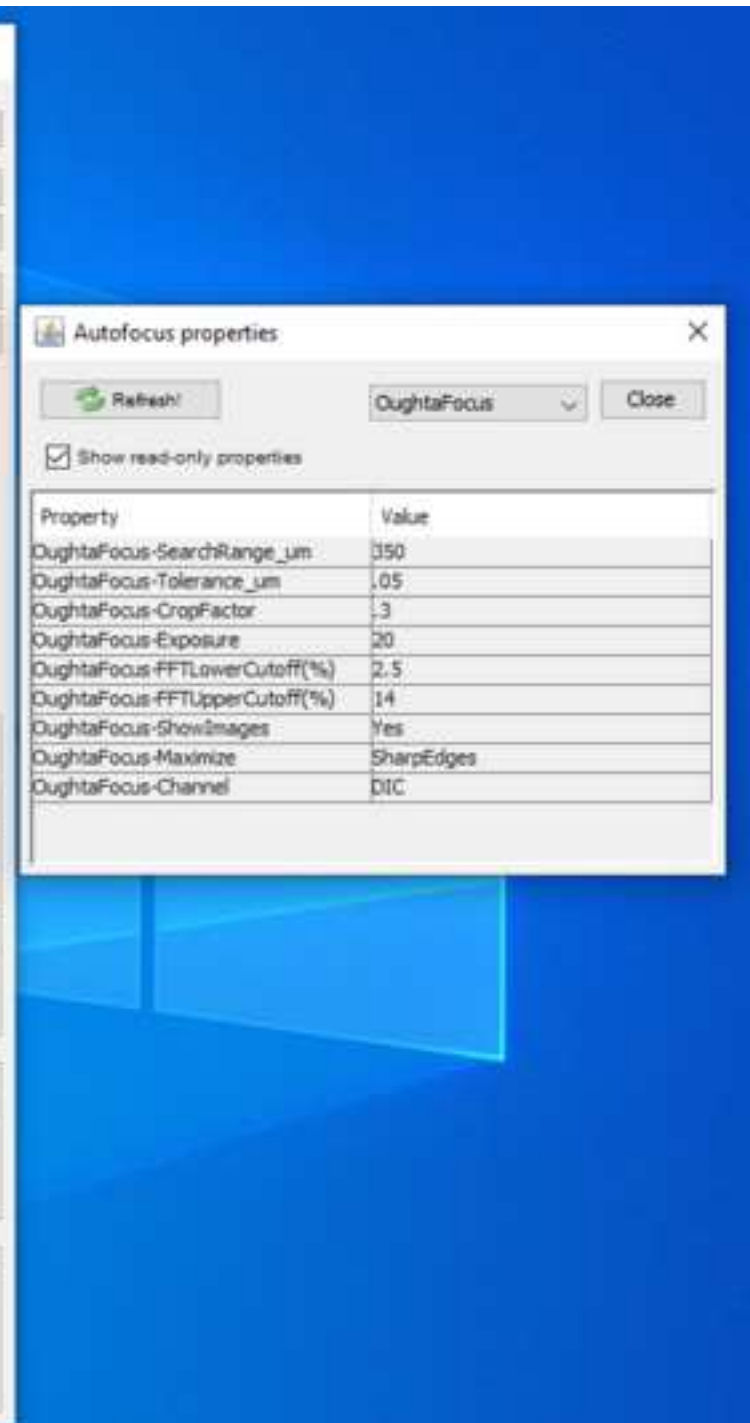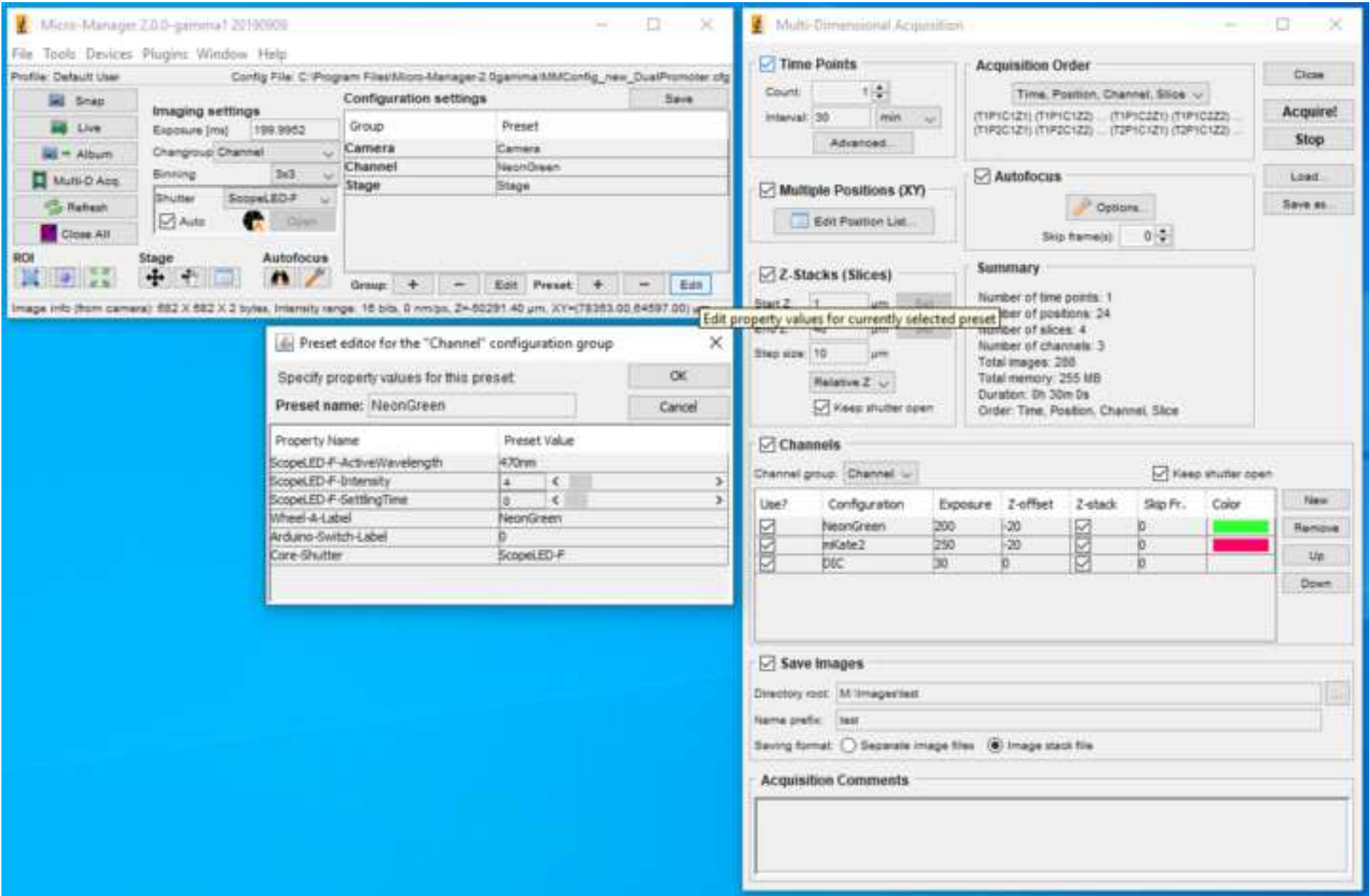
Screen shot 9

Screen shot 12

☑ **Channels**

Channel group: | Channel ⌄ | ☑ Keep shutter open

Use?	Configuration	Exposure	Z-offset	Z-stack	Skip Fr.	Color	
☑	NeonGreen	200	-20	☑	0	🟩	New
☑	mKate2	250	-20	☑	0	🟥	Remove
☑	DIC	30	0	☑	0		Up
							Down

Screen shot 13

☑ **Save Images**

Directory root: M:\Images\test

Name prefix: test

Saving format: ○ Separate image files   ◉ Image stack file

Acquisition Comments

A1: Untreated
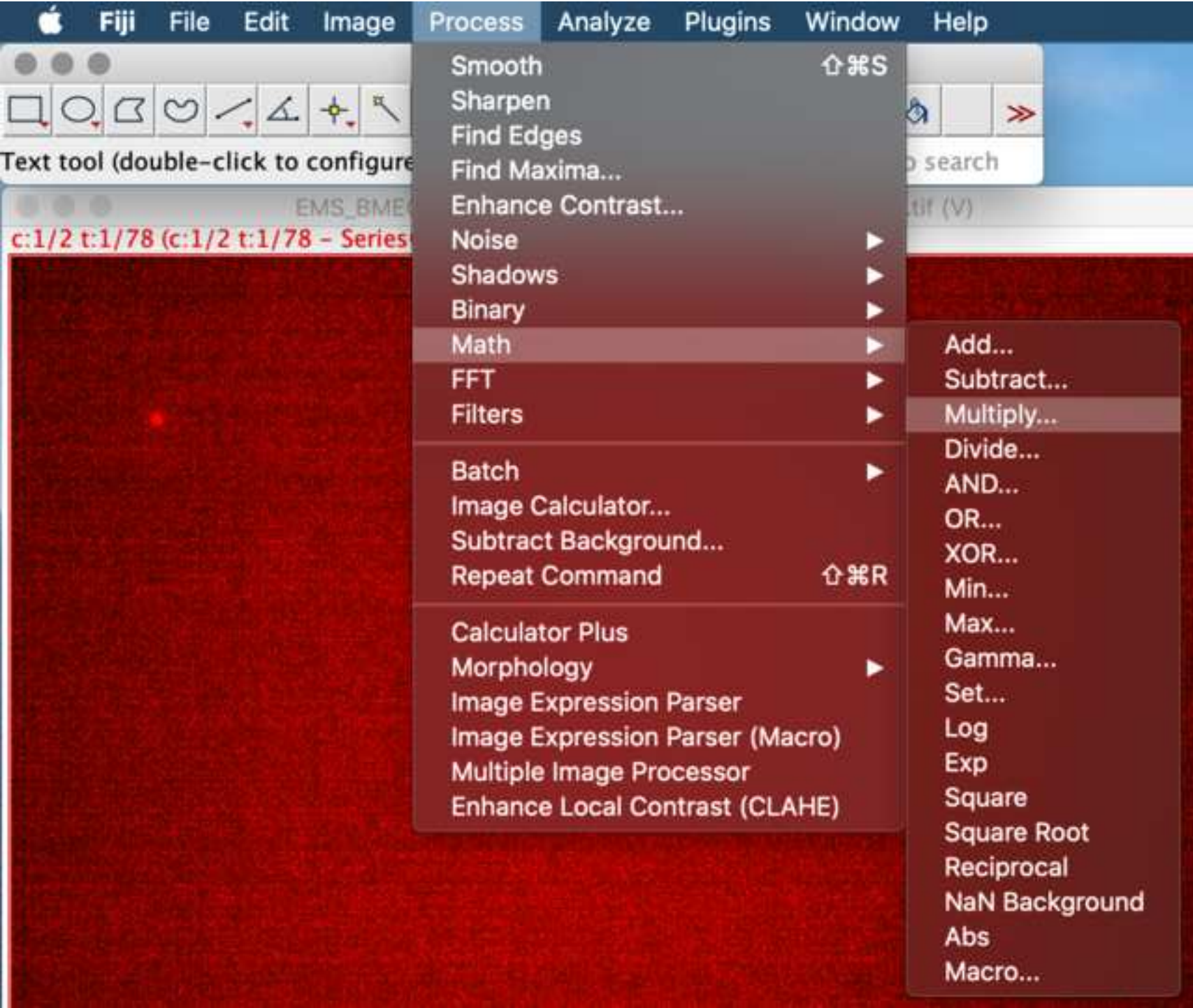A2-3: EMS
B1-3: EMS
Imaging: 12-36 HPI

Subtract Background...

Rolling ball radius: 50.0 pixels

☐ Light background

☐ Create background (don't subtract)

☐ Sliding paraboloid

☐ Disable smoothing

Help   Cancel   OK

Screen shot 22

TrackMate v5.2.0

Settings for detector:

LoG detector

This detector applies a LoG (Laplacian of Gaussian) filter to the image, with a sigma suited to the blob estimated size. Calculations are made in the Fourier space. The maxima in the filtered image are searched for, and maxima too close from each other are suppressed. A quadratic fitting scheme allows to do sub-pixel localization.

Segment in channel: 1

Estimated blob diameter: 48.000   pixel

Threshold: 0.000

☐ Use median filter

☑ Do sub-pixel localization

🏁 Preview

💾 Save   ⓘ 🔧   ⬅ ➡ Next

TrackMate v5.2.0

Settings for tracker:

Simple LAP tracker

This tracker is identical to the sparse LAP tracker present in this trackmate, except that it proposes fewer tuning options. Namely, only gap closing is allowed, based solely on a distance and time condition. Track splitting and merging are not allowed, resulting in having non-branching tracks.

Linking max distance: 8.000   pixel

Gap-closing max distance: 8.000   pixel

Gap-closing max frame gap: 1

Save   ℹ️   🔧   ⬅   ⮕ Next

Screen shot 27
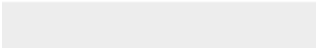
Spots in tracks statistics

Label	ID	TRACK_ID	QUALITY	POSITION_X	POSITION_Y	POSITION_Z	POSITION_T	F
ID3164	3164	869	0.483	218.726	499.497	0	7	7
ID9728	9728	869	0.392	221.091	492.409	0	8	8
ID8144	8144	869	0.376	223.253	491.764	0	9	9
ID11254	11254	869	0.331	215.848	491.598	0	10	1
ID12067	12067	869	0.441	218.746	494.640	0	11	1
ID8970	8970	869	0.412	216.157	493.816	0	12	1
ID7417	7417	869	0.463	222.095	488.856	0	13	1
ID6654	6654	869	0.476	218.818	494.595	0	14	1
ID10499	10499	869	0.422	218.086	494.460	0	15	1
ID12818	12818	869	0.512	215.829	499.381	0	16	1
ID15069	15069	869	0.433	216.953	499.760	0	17	1
ID13559	13559	869	0.489	218.337	502.369	0	18	1
ID14294	14294	869	0.571	216.565	503.108	0	19	1
ID15844	15844	869	0.569	215.095	506.125	0	20	2
ID16593	16593	869	0.494	214.175	504.941	0	21	2
ID17278	17278	869	0.809	218.632	508.468	0	22	2
ID18016	18016	869	0.763	217.418	510.601	0	23	2
ID18833	18833	869	0.996	216.481	512.799	0	24	2
ID19528	19528	869	1.120	214.336	518.794	0	25	2
ID20939	20939	869	1.353	211.735	524.215	0	26	2
ID20208	20208	869	1.470	208.211	525.861	0	27	2
ID21646	21646	869	1.646	207.331	529.476	0	28	2
ID1668	1668	995	0.373	493.130	329.622	0	6	6

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Reduce_Z

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Reduce_Z_kertosis_2ch_JOVE.py

TrackMate