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Multi-Position Photoactivation and Multi-Time Acquisition for Large-Scale Cell Tracing in Avian Embryos

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INTRODUCTION

Vertebrate development is a complex orchestration of cell and tissue movements. Tracing individual cell positions can rapidly become a large-scale problem since cell numbers often grow exponentially in the early embryo. A typical approach consists of fluorescently marking small numbers of cells within a large number of embryos, followed by comprehensive 3D static or time-lapse imaging to map cell positions. However, for large-scale cell tracing, such as during organogenesis, this approach has limitations in time and effort and expensive microscopy costs. Multi-position acquisition is a method that can be used to capture more than one location on the microscope stage and allow for multi-specimen imaging. When multi-position imaging is combined with photoactivation cell labeling, a tool for selective cell marking using laser excitation, it offers a powerful tool for rapid data acquisition. Here, we present a protocol for this technique and demonstrate a practical application to map cell movements in the chick spinal cord, using slice culture explants. We describe details of multiple slice culture preparation, multi-position photoactivation, and multi-time acquisition. We accomplish the coordination of these events by setting up blocks of microscope instructions that execute sequentially. We find that this method significantly decreases time, effort, microscopy and embryo costs by a factor of the number of specimens imaged per session, typically by a factor of 6.

RELATED INFORMATION

MATERIALS

Reagents—B27 supplement, serum free (Gibco, 17504-044)

10% India ink (Pelikan Fount, Drawing Ink A, #17 black), prepared in Ringer's solution

Ethanol (70%)

Fertile White Leghorn chick eggs incubated at 38°C in a humidified egg incubator (VWR, WL51475)

Fibronectin, 20ug/ml in phosphate buffer (Gibco, 33016-015)

Neural basal medium (Gibco, 21103-049)

Photoactivatable Fluorescent Proteins (PAFPs): PSCFP2 (Evrogen International)

Ringer's Solution

Equipment—Dissecting microscope (Leica, MZFLIII)

Electroporator ECM 830 (BTX, a division of Genetronics, San Diego, CA)

Filter paper (Whatman, 1001185)

Forceps and Scissors (Fine Science Tools; 11252-30 & 14060-10) CO₂ exchange

Glass bottom petri dishes (MatTek Corporation, P35G-1.5-14-C) (Fig. 1C)

Glass capillary tubes, borosilicate with filament (Sutter; BF100-50-10; Novato, CA)

Glass needle puller (Sutter Instrument, model P-87)

Heated chamber around microscope (XL LSM S, Pecon; Germany)

High vacuum grease (Dow Corning, 59344-05)

Laser scanning confocal microscope (for example, Zeiss LSM 510) with a motorized stage and 405nm laser line on a fluorescent compound microscope (Zeiss Axiovert) (Fig. 1A) (Zeiss, Thornwood, NY, USA)

Micro cover glasses, 25 mm round No. 1 coverslips (VWR, 48380-080)

Microinjector (Picospritzer III, Parker Hannifin Corporation; Fairfield, NJ)

Micropipette microloader (Eppendorf AG, 5242-956-003)

Millicell culture inserts (Millipore, PICMORG50) (Fig. 1C)

MultiTime macro version 4.0.23b (Zeiss)

Needles, 18 & 25 gauge (Becton Dickinson) and syringes, 1 & 5 ml (VWR)

Parafilm (VWR, 52858-076)

Petri dishes (Becton Dickinson, 35×10mm and 100×15mm)

Plastic transfer pipets (Samco, 202–205)

Platinum electrodes (A-M Systems, Inc., #711000)

Razor blades

Tape (e.g. Scotch box sealing tape, 311 clear; 3M)

Tungsten needle (A-M Systems, 717000)

Tungsten needle holder (Fine Science Tools, 26016-12)

Vibratome (Leica, VT-1000S)

METHOD

Preparation of reagents and injection equipment (~20min)

1. Mix 1 ml of India ink and 9ml of Howard Ringer's solution in a 15-ml tube.
2. Prepare a glass needle from a glass capillary tube using a needle puller with the following settings: heat, 400; pull, 50; velocity, 50; time, 100.
3. Load the glass needle with the PAFP construct (concentration of 2.5-5 ug/uL) using a micropipette microloader and a 20 ul pipet.
4. Gently trim the tip of the glass needle such that the PAFP is able to pass through the tip at small quantities with the application of the picospritzer. To do this, bring the tip of the glass needle into gentle contact with the tip of a pair of Dumont # 5 forceps. Tap the picospritzer foot pedal to visualize the amount of dye exuding from the tip of the needle. The picospritzer settings need to be appropriate for the tissue to be injected. For a Hamburger and Hamilton (HH, 1951) stage 10 embryo, the recommended settings for microinjection into the hindbrain neural tube are pressure, 10 psi; duration, 10 ms.
5. Set the electroporator to desired values. For an HH stage 10 embryo and platinum electrodes, the recommended settings are: voltage, 20V; pulse length, 45; pulses, 5.

Preparation of embryos for microinjection of PAFPs (~90min; 5 min/egg)—*The time requirement for this procedure is dependent on the manipulations to be performed.*

6. Incubate fertilized White Leghorn chicken eggs at 38° C in a humidified incubator until the desired stage for manipulation.
7. Rinse the eggs with 70% ethanol. Remove 3 ml of albumin from the wider end of the egg using an 18 gauge needle and a 5-ml syringe. Discard the albumin.
8. Cover the dorsal surface of the egg with 2 pieces of transparent tape placed adjacent to one another so that the tape spans about 2/3 of the length of the egg.
9. Cut a circular window through the tape in the top of the horizontally lying egg. The window size should be large enough to angle the glass needle for microinjection and accommodate the electrodes for electroporation. The tape reduces the amount of eggshell pieces that crack and enter the egg when cutting into the egg.
10. Using a 1 ml syringe and a 25 gauge needle bent at 90 degrees, inject ~0.1 ml of the 10% India ink solution (just enough to provide contrast) directly under the embryo for visualization.
11. Place a few drops of Howard Ringer's solution around the circumference of the embryo with a transfer pipette to prevent drying.
12. With a sharpened tungsten needle, carefully penetrate the vitelline membrane to the side of the embryo and pull it away from the potential injection site using a pair of forceps.

Microinjection and Delivery of PAFPs (~5 min/egg for microinjection; egg re-incubation time may be 5–6hrs)—*The time requirement for this procedure is dependent on the number of embryos to inject and electroporate and also the egg re-incubation time required.*

13. Position the micromanipulator next to the egg and direct the injection needle to the injection site. Inject the PAFP into the selected region.

- 14 After microinjection of the PAFP construct, quickly retract the injection needle and place the electroporator electrodes at the edge of the blastoderm. Place the positive electrode on the right side of the embryo (as viewed from above the egg) and the negative electrode on the left side of the embryo.
- 15 Start the electroporation process using the settings recommended in step 5.
- 16 Add a few more drops of Ringer's solution afterward around the embryo with a transfer pipette.
- 17 Tape over the eggshell window and place the egg back into the 38° C incubator for at least 5–6 hrs before photoactivation, depending on the maturation of the desired PAFP, and the stage of development of interest.

Media and Millicell Insert Preparation (30min)

- 18 Prepare neural basal medium (10 ml), by adding B27 at (200ul) and L-glutamine at (1mg), and place in 37° water bath for 10 minutes.
- 19 While the media is warming up, remove a Millipore filter from its package and coat the surface with fibronectin (~200ul). Let stand covered for 10 minutes at room temperature, with the Millipore filter slightly tilted.
- 20 Remove excess fibronectin by pipetting from the wall of the filter and coat the membrane with neurobasal media until ready to use. Use caution not to contact the membrane surface with the pipet, since this may rip the membrane.

Tissue Preparation (30min)

- 21 When desired stage of development has been reached (post embryo-labeling), re-open the egg by cutting through the tape.
- 22 Screen embryos for health and robust labeling in region of interest. Remove selected embryos from the eggs and place in a Petri dish filled with PBS on ice.
- 23 Using a sharpened tungsten needle, remove the vitelline membrane and excise region of interest (e.g. trunk region). Transfer region of interest to a new Petri dish with fresh Ringer's solution.
- 24 To cut transverse sections, lay the tissue on its side (i.e. dorsal side right, somites up), and position forceps on either side of the explant, gently squeezing the forceps so that they just come in contact with the tissue to hold it in the place.
- 25 Using a fine razor blade (e.g. feather blade), cut transverse sections through the region of interest.
- 26 For better morphology of the cut tissue sections, it is possible to embed the embryo trunk in low melting point agarose, mixed with neural basal culture media (from step 18). The agarose can be trimmed to remove excess agarose down to near the tissue of interest and then superglue the agarose block to the stage of the vibratome for sectioning. The tissue sections can be cut in 100um sections (for trunk) at a speed of 5, frequency of 8. With a paintbrush, each tissue section is ready to transfer to the Millipore culture insert (Fig. 1K).

Culture and petri dish assembly (1hr)

- 27 Place 1ml of supplemented neurobasal medium into a glass-bottom Petri dish and place the Millipore filter insert from above (pre-coated with fibronectin) into the Petri dish.

- 28 Gently transfer the transverse sections onto the Millipore filter using a plastic transfer pipet with a large diameter (i.e. trim the end of the pipet to generate a larger diameter opening to transfer tissue with).
- 29 Use forceps to gently orient the transverse sections on the Millipore filter to fit within the glass imaging circle on the bottom of the Petri dish.
- 30 Remove excess liquid from the surface of the Millipore filter so the tissue sections lay flat. Remove excess liquid from inside the Petri dish as well so that the filter is sitting against the bottom of the Petri dish.
- 31 Place the lid on the Petri dish and seal it with a piece of Parafilm.
- 32 Place the dish on the microscope stage within the heated chamber and allow the dish to equilibrate to 37°C (~30 minutes) (Fig. 1B).

Heated chamber calibration: *The time requirement is dependent on controller and temperature of room. Unless the system is changed (temperature or components) or the microscope is within a core facility, calibration need only be performed once. The heated chamber can be self-built (see Kulesa and Kasemeier-Kulesa, CSH Protocols; 2007; doi: 10.1101/pdb.prot4792) or commercially purchased.*

- 33 Insert the probe of a digital thermometer within the heated chamber (Fig. 1A). Tape the probe onto the microscope stage.
- 34 Turn on the heated chamber until the temperature at the stage is maintained at 38°C.
- 35 Record the time to reach equilibrium. This should be factored into the culture preparation to limit the time the embryo or tissue is out of the incubator.
- 36 Let system equilibrate 30 minutes before imaging.

Photoactivation of Dual Color Fluorescent Forms (e.g., PS-CFP2) Imaging Set-Up: Establish positions to be imaged (~4 min / location)—All locations have to be configured before image acquisition is started.

- 37 Move the microscope stage to view desired sample position. (0,0) is set by the microscope.
- 38 Configure all the imaging parameters desired to acquire images before photoactivation, including image rotation (Fig. 1G), z-stack settings (Fig. 1H), scan speed, laser power, gain, offset, frame averaging, bit depth, and resolution. These should be set-up for the brightfield image and PAFP prior to photoactivation.
- 39 Save the configuration and note the position for which it applies.
- 40 Mark this position in the stage control. For optical z-sectioning, the marked location should be made from the middle of the z-stack.
- 41 Repeat steps 36–39 for the desired number of positions.

Imaging Set-up: Photoactivation cell labeling

- 42 Move stage to first location to be photoactivated.
- 43 To target a subpopulation of cells for photoactivation of a dual color fluorescent form, such as PSCFP2, zoom in on a particular region of interest using the optical zoom. To select cells for photoactivation cell labeling, crop the region of

excitation and pre-scan the region of interest (Fig. 1I-J). The selected cells do not have to be completely located inside the cropped region of interest before photoactivation, since photoactivation cell labeling of the cytoplasm will transfer throughout the cell.

- 44 To photoactivate PSCFP2 within the region of interest, use a 405-nm laser with a power setting between 15–20%. When scanned using a single image scan, the 405-nm laser will photoactivate the PSCFP2 within the cell. A single scan may not be enough to optimize the photoactivation. However, remember that as the image is zoomed in, the power from the laser increases: $\text{laser dosage} = (\text{laser intensity} \times \text{scan time}) / \text{scan area}$, so care should be taken to photoactivate, but not photobleach the cells of interest. As a guideline, the optical parameters for PSCFP2 photoactivation in the chick embryo has been previously reported (Stark and Kulesa, 2007).
- 45 To evaluate the increase in mean PSCFP2 fluorescence in the region of interest, follow-up the 405-nm excitation scan with a 488-nm excitation scan. Monitor the photoefficiency of the photoactivation process by measuring the fluorescence intensity changes within the region of interest following the 488-nm excitation scan, using a line intensity profile comparison.
- 46 When photoactivation is complete in the region of interest, measured by the maximum amount of fluorescence intensity before photobleaching, save the configuration, noting location.
- 47 Move the stage to the next location (Fig. 1L,M).
- 48 Repeat steps 42–44 until all locations have a photoactivation setting saved.

Imaging Set-up: Configure the software for block setting execution

- 49 In order to automate the series of commands for multi-position photoactivation and multi-time acquisition, it is necessary to set up block functions in multi-function software. We have utilized the MultiTime macro, available from Zeiss and have written this section with general terms that may be applicable to other multi-function microscopy software.
- 50 The first mode of acquisition is to collect an image prior to photoactivation. The software must be told to pull information from the microscope stage and configuration controls. In the MultiTime macro, this is performed through the 'Update' button (the function of the 'Update' button is to pull the location (see example in Fig. 2A,A') and configuration (Fig. 2D,D') information from the microscope into the MultiTime macro).
- 51 It is necessary to set the imaging priorities for the experiment. In MultiTime, this is performed by selecting the "Group Rotation –Group –Location" button, or "GR-G-L." (Fig. 2C). This sets the group rotation (that is, the rotation between each of the tissue slice positions) to have the highest priority and to finish the time point on every location before moving on to the next rotation, as opposed to finishing the time lapse on the current location before moving on to the next location. A 'Group' is defined by a set of blocks (Fig. 2B) and 'Location' is defined as a position (Fig. 2A,A') where the microscope performs the block functions (Fig. 2C,D).
- 52 Depending on the multi-position software being used, each location can utilize a different imaging configuration. Set the correct configurations (from step 39) for each location (Fig. 2A,A'), including z-sectioning, if desired (Fig 2D, D').

- 53 To add an event or special imaging technique to the experiment, a second mode of acquisition needs to be conveyed to the software. This is performed in MultiTime by creating a second block in the experiment (Fig. 2B). This will be used to photoactivate the regions of interest (see example in Fig. 1L,M).
- 54 Set the correct photoactivation configurations (Fig. 2D,D') for each location (Fig. 2A,A') in this second block (from step 46).
- 55 If more than one scan is required to photoactivate the PAFP of interest (as mentioned in steps 44–45), this should be configured now. This is adjusted in MultiTime using “number (No.) of scans” to set the configuration (Fig. 2D,D').
- 56 Now the software is set to image, and then photoactivate each desired region of interest. To image post-photoactivation, create a third block in the experiment (Fig 2B,B') and set its configurations correctly for each location for imaging (from step 39) (Fig. 2D,D') and not photoactivation (from step 46)
- 57 If a time-lapse is desired, this is the appropriate place to configure the software. Time-lapse experiments in MultiTime have to be configured by repeating the acquisition a set number of times with a fixed time interval between frames, or “group repetition” with a “wait delay” (Fig. 2C). The software default is to place a time delay between the end of the first acquisition (at position 1) and the beginning of the next acquisition (at position 2). If no interval is specified, the macro will acquire all images continuously; one position to the next. Also, if a time-lapse is desired, the third block will have to be its own group since MultiTime has no block repetition (only group repetition).
- 58 Save the images in the desired location in either single database or multiple databases format, depending on how the images are to be organized. Choosing multiple databases allows each location (specimen) to have its own database folder and simplifies manipulation and image processing.
- 59 Start acquisition.

TROUBLESHOOTING

Problem: Photobleaching occurs during the photoactivation process. [Step 44]

Solution: Photobleaching typically occurs after the maximum photoefficiency of the PAFP has occurred. This can be prevented by using a low laser power setting or an objective with a low magnification, such as a 10X objective. A lower magnification objective will deliver less laser energy to the region of interest and allow for tighter control to maximize photoefficiency, but prevent photobleaching. As the image is zoomed in, the power from the laser increases by: $\text{laser dosage} = (\text{laser intensity} \times \text{scan time}) / \text{scan area}$. The higher the zoom, the less percent laser power is required. It is recommended to have 2–3% laser power if zoom is >35X and 3–5% laser power if zoom is <34X. If immediate photobleaching occurs, the 405nm (or photoactivatable excitation wavelength) laser power is too high and must be lowered accordingly for the subsequent regions of interest. Photoefficiency should be monitored during photoactivation to achieve maximum photactivation without photobleaching.

Problem: Depending on the efficiency of the photoactivation and movement of photoactivated cells deeper in the tissue slice, photoactivated cells may become less distinct amongst the host population over time. [Steps 44–59]

Solution: Photoactivated cells can be reactivated following the same procedure as the original photoactivation.

Problem: Cells that were not directly targeted appear photoactivated prior to photoactivation step. [Step 43]

Solution: Inadvertent photoactivation can result from a mercury lamp source during the initial survey of PAFP-expressing cells. All focused light should be avoided until the sample is prepared for the microscope stage. Control tissue should be used to determine the extent of inadvertent photoactivation due to mercury lamp source use.

Problem: Neighboring cells to the photoactivated region appear photoactivated. [Steps 43,45]

Solution: If the goal is to label an individual cell, 1-photon confocal laser scanning excitation may provide too much out of focus excitation light and inadvertently photoactivate neighboring cells above or below the target of interest. Using 2-photon excitation will limit the amount of out of focus laser excitation and significantly limit inadvertent photoactivation.

Problem: Experiment has a time delay between photoactivation and start of acquisition. [Step 57]

Solution: The delay between time points occurs before everything else in the Block setup instead of after it. If using MultiTime, insert a new block with settings identical to the final one between the photoactivation block (second) and post photoactivation imaging block (third). The new block should have no delay or repetitions for time-lapse.

Problem: The computer freezes up when concatenating data after all images are taken. [Step 59]

Solution: The final file size is too large and the imaging computer cannot handle all of the processing. Concatenation of the images can be done on a different machine. If using MultiTime, uncheck “save final images” in options and use the modify series macro to concatenate the images post-acquisition. MultiTime saves the images from each position in a “temp_[location number]” folder.

Problem: Time delay is being measured from end of one time point to start of next rather than start of one to start of next. [Step 57]

Solution: The software default is to place a time delay between the end of the first acquisition (at position 1) and the beginning of the next acquisition (at position 2). If no interval is specified, the macro will acquire all images continuously; one position to the next. Time delay settings need to be changed to interval. This is done in MultiTime in the options menu by changing “delay” to “interval.” This will delay from beginning of one repetition to the beginning of the next.

Problem: A sample has moved out of focus during time-lapse. [Step 59]

Solution: There are two options, if the out of plane movement does not involve drift. The first option is to pause the acquisition before the next out of focus position is acquired and turn the focus knob to put the sample back in focus. Resume acquisition. Since only the starting positions are saved and cannot be updated during acquisition, it will be necessary to re-focus each this position is repeated. The second option is to stop the acquisition and re-configure to set the updated position after re-focus.

Problem: Dilution of photoactivated fluorescence signal due to cell proliferation or degradation of photoactivated molecules. [Step 42]

Solution: We found that there was minimal loss in photoactivated fluorescence signal (~5%) after cell division (Kulesa et al., 2008). Using photoactivation cell labeling in embryos is effective for periods up to 48hrs (Stark et al., 2007), with cell cycle time on average of 9–10hrs. However, if cell cycle time is rapid and long-term time-lapse imaging is required, it may be necessary to consider an alternative method for fluorescent cell marking or re-photoactivation of regions of interest.

Problem: Time-lapse cannot acquire all desired positions in the preferred time point interval. [Step 57]

Solution: There are a few options from which to choose depending on experimental requirements. One, choose a faster scan speed. Two, shorten the amount of images you are acquiring, either by fewer images in the z-stack or fewer positions altogether. Three, make your time point interval longer.

DISCUSSION

We have presented a technique for large-scale cell tracing in embryogenesis, using a combination of multi-position photoactivation and multi-time acquisition. We have applied the technique to trace cell positions in the chick spinal cord, recording fluorescently labeled cell movements in a number of transverse tissue slices. By multiplexing the number of regions of interest in multiple tissue slices taken from the same embryo, the technique overcomes the roadblocks of time, effort and microscopy and embryo costs associated with large-scale cell tracing. For example, by using an average of 6 slices per experiment, we were able to cut our imaging time and costs by a factor of 6. Since microscope time (widefield, confocal or two-photon) typically ranges between \$25–\$75/hr, and chick embryo costs can be \$2/egg, reducing our costs by a factor of 6 represented significant savings (~\$20K).

We combined multi-time acquisition in a number of samples with a separate event in acquisition, specifically photoactivation. We set up the photoactivation to sequentially cover neighboring regions of interest in the chick spinal cord, with a dorsal-to-ventral height of ~50um and width of the half-neural tube. The imaging parameters described above may be set and modified to accommodate multiple, distinct regions of photoactivation and multi-position imaging of tissue or embryos, depending on the anatomical structures of interest. Our typical time-lapse imaging session was approximately 12hrs, which is within 24hr range of the slice explant culture we have previously developed. We did not observe changes in tissue morphology and anticipate that this will be a function of the tissue thickness, type, and culture method used in each application. Embedding the tissue or embryo in agarose before setting up a slice culture explant may sustain the tissue morphology for long-term time-lapse imaging.

We found that the most versatile of the parameters was the location and size of the regions of interest at each position. The ROI can be set to include the entire field of view, a subpopulation of cells or an individual cell. In our example, we visually determined the neighboring regions of interest, such that there was minimum overlap between successive tissue slices. Unfortunately, this method had no simple way of pre-determining where the region of interest from an individual tissue slice was relative to the region of interest in the next slice. Also, we were limited to using rectangular regions of interest, even though the

natural boundaries of the tissue were curved. Future work to develop an algorithm to more efficiently cover a given specimen shape would be very helpful.

We based our protocol and description of events on use of a Zeiss microscope platform and Aim software, but attempted to make the multi-position directions as general as possible in order to be applied to other microscope software. For example, the Leica SP5 Microscope with LiveDataMode software package will accommodate this procedure as will the Olympus MicroSuite FIVE Advanced Suite (http://www.leicamicrosystems.com/fileadmin/downloads/Leica%20TCS%20SP5%20II/Application%20Notes/LiveDataMode-AppLetter_27.EN.pdf) (http://www.olympusamerica.com/seg_section/msfive/ms5_advsuite.asp).

It was important to keep in mind or estimate how many images were acquired in a typical multi-position photoactivation and multi-time acquisition time-lapse session. We found that optical sectioning of each tissue slice, combined with up to 6 tissue sections significantly multiplied the amount of collected data. For example, on an overnight time-lapse imaging session of 6 tissue sections, we averaged 12GB of data (2GB per tissue section). We recognize that the most time intensive component of this type of project is data analysis and remains a challenge for the field.

Multi-time acquisition may be combined with multiple, separate fluorescence events, such as photoactivation, and sequentially performed to address a specific biological question. Although we applied this technique to the question of large-scale cell tracing, it is adaptable to a wide variety of experiments with minor alterations to the parameters and by re-defining the block function and sequential acquisition format. Thus, events such as tiling, FRAP, FRET, and FLIP may be combined with multi-position acquisition to create a powerful tool to reduce the time and costs required for more complex experiments typically performed in a single embryo sample.

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Transmitted light has been removed and an outline of the neural tube has been added. The scale bar is 100 μm .

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Figure 2. Configuration of MultiTime macro

(A) Position section of MultiTime macro. (A') Saved stage positions corresponding to tissue sections are viewable in a drop down menu when imported with 'Update'. Each position can be given a different configuration. (B) Block setup section of the macro. Block setup determines the sequence of events. Each block is configurable to execute specific microscope settings in conjunction with the position highlighted. Blocks can also be joined into groups. Each block here is in its own group. (B') Block 1 will take a single image of the field of view to get a baseline intensity for the PAFP of interest. Block 2 will scan the specified Region of Interest 8 times (specified in (D): 'No. of scans') to photoactivate the PAFP in that area. Block 3 executes the time-lapse parameters specified in (C) to be used in

acquisition. (C) Block properties section of macro. Experiment settings and individual block settings are configured here. Shown is a setup for taking 18 time points with a 10 min delay between each. (C') Imaging priority is crucial as it will dictate the order in which the images are acquired. (D) Microscope configuration section of macro. (D') Saved configurations must be specified for each position, for each block. They are listed in a drop down menu when imported with 'Update'. (D'') If a z-stack or other special function is desired that must be checked here.

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