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## Clonal Genetic Tracing using the Confetti Mouse to Study Mineralized Tissues

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Vineeta Bajaj, PhD  
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## Cover Letter

Dear Dr. Bajaj,

Thank you for your comments and for giving us an extension to the resubmission deadline. We have streamlined the protocol section, refocused the representative results and edited the figure/video legends as you advised.

Please find the updated version of the manuscript entitled "Clonal genetic tracing using the Confetti mouse to study mineralized tissues", a "Rebuttal letter" attached, and new versions of "Figure 1" and "Supplementary File 1".

Thank you once again,

Best wishes,

Phillip Newton, of behalf of all authors

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**TITLE:**

Clonal Genetic Tracing using the Confetti Mouse to Study Mineralized Tissues

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**KEYWORDS:**

R26R-Confetti, Confetti, Brainbow, growth plate, cartilage, bone, articular cartilage

**SUMMARY:**

This method describes the use of the R26R-Confetti (Confetti) mouse model to study mineralized tissues, covering all steps from the breeding strategy to the image acquisitions. Included is a general protocol that can be applied to all soft tissues and a modified protocol that can be applied to mineralized tissues.

**ABSTRACT:**

Labeling an individual cell in the body to monitor which cell types it can give rise to and track its migration through the organism or determine its longevity can be a powerful way to reveal mechanisms of tissue development and maintenance. One of the most important tools currently available to monitor cells in vivo is the Confetti mouse model. The Confetti model can be used to genetically label individual cells in living mice with various fluorescent proteins in a cell type-specific manner and monitor their fate, as well as the fate of their progeny over time, in a process called clonal genetic tracing or clonal lineage tracing. This model was generated almost a decade ago and has contributed to an improved understanding of many biological processes, particularly related to stem cell biology, development, and renewal of adult tissues. However, preserving the fluorescent signal until image collection and simultaneous capturing of various fluorescent signals is technically challenging, particularly for mineralized tissue. This publication describes a step-by-step protocol for using the Confetti model to analyze growth plate cartilage that can be applied to any mineralized or nonmineralized tissue.

## INTRODUCTION:

Improved methods to monitor cell behavior are desirable to increase the experimental efficiency when using animal models. One of the most informative approaches to monitor cell behavior in vivo is clonal lineage tracing with multicolor reporter mouse strains<sup>1</sup>, including the widely used R26R-Confetti (Confetti) mouse<sup>2</sup>. By genetically labeling individual cells with fluorescent proteins and following those cells over time, researchers in many fields have used the Confetti mouse to reveal insights into a multitude of different biological systems.

The Confetti mouse is a loxP-based reporter system in which Cre dependent DNA recombination causes the permanent expression of one of several possible fluorescent proteins in a stochastic manner<sup>2</sup>. The R26R-Confetti allele includes the ubiquitously expressed CAGG promoter, which lies immediately upstream of a loxP-flanked Neo<sup>R</sup>-cassette<sup>2</sup>, whose polyadenylation sequence terminates transcription<sup>3</sup>, followed by the Brainbow 2.1 construct<sup>4</sup>. Hence, Cre-mediated recombination simultaneously excises the Neo<sup>R</sup>-cassette and leads to the production of certain fluorescent proteins depending on which parts of the Brainbow 2.1 construct are excised. This feature makes the Confetti mouse extremely adaptable because it can be used to target any cellular population in a mouse for which a specific Cre strain is available. The crossing of a tissue-specific Cre strain with the R26R-Confetti strain provides the specificity of labeling. However, the Cre strain should also be inducible (e.g., CreERT or CreERT2, which require tamoxifen binding to allow them to enter the nucleus and induce DNA recombination<sup>5</sup>) so that labeling can be achieved at specified time points. Thus, a cellular population can be labeled by injecting the resultant CreERT-positive/Confetti-positive offspring with tamoxifen at the desired time point and tracked to a certain age, when the tissues of interest can be collected for analysis. After tissue processing, the fluorescently labeled cells can be directly visualized by confocal microscopy so that the progeny of each initially-labeled cell can be separated from the progeny generated by any other labeled cell based on their specific fluorescent labels, thereby allowing clonality to be assessed (i.e., clonal genetic tracing).

Due to the flexibility of the Confetti model, it has been applied to study the development and maintenance of many different tissues in health and disease<sup>2,6-11</sup>. One common way to use the model is to label a certain population of cells and determine which tissues they (and/or their progeny) have contributed to. One such finding using this approach was that the adult tooth is comprised of cells with a glial origin<sup>6</sup>. Another application of the model is to study stem cell homeostasis. For example, the Confetti model revealed that stem cell niches in the intestinal crypts gradually become monoclonal because some stem cell clones are dominant during competition between stem cells<sup>2</sup>. The model can also be used to assess cell proliferation, which is particularly useful for studying slowly dividing cells. For example, clonal formation in the articular cartilage<sup>12</sup> was assessed, and the short-term effects of a hedgehog antagonist on growth plate chondrocytes in five-week old mice was studied<sup>13</sup>.

Despite the relative simplicity of the Confetti model, the fluorescent signal can be technically challenging to preserve until image collection, particularly when analyzing mineralized tissues. Here, the protocol describes an optimized model to use the Confetti mouse with the postnatal bone (up to 6 months of age), enabling fluorescent proteins to be directly visualized by confocal

microscopy without the need for immunodetection. This simplified protocol can be applied to nonmineralized tissues. Furthermore, included is a description of how to store the samples in order to preserve the fluorescent signal for a prolonged period of at least 3 years. An outline of the protocol is presented in **Figure 1**.

## **PROTOCOL:**

All mouse work was approved by the Ethical Committee on Animal Experiments (Stockholm North Committee/Norra Djurförsöksetiska Nämnd) and conducted in accordance with The Swedish Animal Agency's Provisions and Guidelines for Animal Experimentation.

### **1. Mouse breeding**

1.1. To generate the strain, cross the R26R-Confetti mouse with the Cre line of interest. Wean the pups at 21 days of age.

NOTE: Mice can be used for breeding from approximately 8 weeks of age, or according to the local guidelines. Genotyping (not described here) can be performed from biopsies collected at weaning. The age at weaning can be adjusted as per local guidelines.

### **2. Confetti labeling**

NOTE: This labeling strategy is appropriate for tamoxifen inducible Cre strains.

2.1. Prepare a solution of up to 2.5 mg/mL tamoxifen in corn oil in a glass bottle. Dissolve by heating to 37 °C in an oven for up to 60 min, agitating every 5 min using a vortex until the powder has dissolved.

NOTE: For increased doses of tamoxifen, solutions up to 20 mg/mL can be prepared using this method.

2.2. Store at 4 °C for up to 3 months, protected from light.

2.3. Induce recombination by administering 50 µL of 2.5 mg/mL tamoxifen dissolved in corn oil intraperitoneally on the first postnatal day (P1).

NOTE: In principle, tamoxifen can be administered at any age, as early as the F1 generation. The dose of tamoxifen should be varied based on Cre expression, mouse age, and experimental application. Further information can be found in the discussion section. Cre negative mice treated in the same way can be used as a negative control for Confetti fluorescent signals.

CAUTION: Ensure animal handlers are warned of the tamoxifen use and ensure that cage materials are disposed of according to local environmental and safety guidelines.

### 3. Tissue collection

3.1. Euthanize mice on postnatal day 27 (P27) by gradually filling the space containing the mice with carbon dioxide up to at least 80% by volume and maintaining this concentration for 3 min. Perform cervical dislocation. Keep mice on ice during the dissection of the other animals.

3.2. Dissect out the tissue of interest. Outlined below is a protocol for collecting the proximal tibial and distal femoral growth plates and articular cartilage of the knee, suitable for postnatal mice up to the age of 6 months.

3.2.1. Remove the skin around the hind limbs as far as the foot, using sharp scissors and toothed forceps.

3.2.2. Roughly trim the muscle and fat tissue from the legs with sharp scissors.

NOTE: Do not clean the bones perfectly, because the surrounding tissues provide some structural support when sectioning, but ensure all skin is removed.

3.2.3. Using a scalpel, cut through the soft tissue where the top of the leg meets the body until the femoral head is visible, then cut the ligaments in the hip joint to remove the leg.

NOTE: Alternatively, if it is not possible to find the femoral head, cut through the femur close to the hip with strong scissors.

3.2.4. Dissect the foot from the rest of the leg using a scalpel or sharp scissors.

### 4. Tissue fixation and processing

NOTE: Based on the tissue of interest, follow one of the two protocols detailed below (4.1 for soft tissues or 4.2 for mineralized mouse tissues over the age of approximately 45 days, as detailed below). For both methods, store the dissected samples in the 3.7% formaldehyde/phosphate buffered saline (PBS) solution on ice while dissecting the remaining animals.

4.1. For soft tissues and mineralized tibiae and femora up to the age of approximately P45, fix the tissue in precooled 3.7% formaldehyde/PBS for 6 h, rolling gently on a rotator at 4 °C. Use a volume at least 10x greater than that of the tissue. Continue directly to step 4.3.

4.2. For mineralized tissues, including the tibiae and femora over the age of approximately P45, a decalcification step is required.

4.2.1. Prepare 1 L of 10% EDTA/dH<sub>2</sub>O solution with the pH adjusted to 8.05 using sodium hydroxide. Next, dilute the formaldehyde to 3.7% using this solution. The working concentration of EDTA will be 9%.

4.2.2. Fix the tissue by placing into precooled 3.7% formaldehyde/PBS overnight, gently rolling on a rotator at 4 °C.

4.2.3. Place the tissue into precooled 3.7% formaldehyde/9% EDTA/dH<sub>2</sub>O solution (pH 8.05), and gently rotate at 4 °C in a volume at least 10x greater than that of the tissue. Repeat this step by placing the bones into fresh, precooled 3.7% formaldehyde/9% EDTA/dH<sub>2</sub>O for 3x over the next 48 h.

NOTE: This short decalcification is enough to allow for sectioning of the femur and tibia bones of up to 6 months-old mice. For more densely mineralized tissues further decalcification may be required to improve histology.

4.3. Transfer the tissue into precooled 30% sucrose/dH<sub>2</sub>O. Make sure to fill the container to the brim, and gently rotate overnight at 4 °C. Use a volume at least 10x greater than that of the tissue. The tissue will often float when first placed into this solution and sink to the bottom of the bottle by the morning.

4.4. To remove the residual sucrose solution, briefly wash the tissue by removing it from the sucrose solution with forceps and completely covering the sample with approximately 5 ml of optimal cutting temperature compound (OCT) for several seconds.

4.5. Fill a prelabeled cryomold with OCT and position the tissue at the bottom. Work quickly to avoid the samples sitting at room temperature for too long.

NOTE: It is important to place the sample in a convenient orientation for the sectioning step. To increase the chances of sectioning through entire growth plate columns after tracing with Col2CreERT:Confetti, place the medial side facing down using the femoral head as a guide, and position the sample as flat to the bottom of the mold as possible.

4.6. Embed the sample by placing the mold onto dry ice and waiting for the OCT to entirely solidify. Place the cryomolds as flat as possible to avoid movement/sliding of the tissue in the mold. Once finished, store the samples at -20 °C, if they are not used immediately.

NOTE: Use within 12 weeks, because with age the blocks become more difficult to section.

## 5. Sectioning

NOTE: Perform cryosectioning using a cryostat with disposable blades suitable for hard tissue. Any standard cryostat is suitable.

5.1. Remove the block from the mold and secure it to the chuck by applying OCT between the top of the block and the chuck and cooling in the cryostat at -20 °C for at least 5 min, so that the OCT has completely solidified.

5.2. Precool both the sample holder and the blade holder to -20 °C and then place the sample/chuck in the chuck holder and the blade in the blade holder to equilibrate for several min.

NOTE: The specified temperatures may be varied by several degrees depending on the cryostat and the tissue of interest.

5.3. Use the cryostat to trim the block and cut tissue sections of a suitable thickness to allow for the visualization of the clones in the tissue of interest.

5.3.1. For postnatal growth plate analysis, prepare sections of 30–160 µm and collect them on slides. Some cryostat models may only allow the collection of thick sections using the trim setting.

5.4. Air dry the slides at room temperature until they are completely dry.

NOTE: The duration of this step can vary based on tissue properties and section thickness. The temperature of the room is also likely to affect the speed of this step.

5.5. Store the slides at -20 °C for up to 36 months, or if processing immediately, proceed to step 6.2.

## 6. Slide preparation and mounting

6.1. Remove the slide from the freezer and bring up to room temperature horizontally in a slide holder. Allow any condensation to evaporate.

6.2. Remove the OCT by using a Pasteur pipette to gently apply PBS at room temperature onto the slide. The thicker the section, the longer time is required. For 160 µm thick sections, perform two rinses: incubate once for 15 min and remove the liquid, and then apply fresh PBS for 5 min and remove the liquid.

NOTE: This step is to remove the OCT and can be varied based on tissue properties and section thickness, as appropriate. The temperature of the room is likely to affect the speed of this step.

6.3. Mount the slides in a solution of 75% 2,2-thiodiethanol/dH<sub>2</sub>O at room temperature with 60 mm long cover slips.

NOTE: Slides can be prepared immediately before imaging, but the fluorescent signal is stable for 1–2 weeks if kept out of the light in a humidified chamber at 4 °C.

## 7. Imaging by confocal microscopy

7.1. Set up the microscope.

7.1.1. Turn on the microscope and open the software. Click the **Start System** button.

7.1.2. Select the channels by first clicking **Smart Setup** under the **Acquisition** tab and then selecting the three channels corresponding to red fluorescent protein (RFP), yellow fluorescent protein (YFP), and cyan fluorescent protein (CFP) under the **Dye** heading. Click **Best signal** then **Apply** to select the lasers.

NOTE: The contents of the **Acquisition** tab are shown in **Supplementary file 1A**. If the required channels are not present, add them using the '+' button under the **Dye** heading. The necessary information regarding laser, excitation wavelengths, and the range of recorded emission wavelengths is shown for the three Confetti fluorescent proteins in **Supplementary file 1B-D**.

7.1.3. Select the 20x objective lens under the **Objective** heading in the **Acquisition Mode** tab.

7.2. Locate the growth plate or other tissue of interest.

7.2.1. To provide a distinct view, first select the RFP channel by clicking its name in the **Channels** tab. This causes details of the RFP channel to be displayed in the **Light Path** tab. Click the tick-box beside **T-PMT**.

NOTE: T-PMT channel shows nonreflected laser light, which is used to visualize the nonfluorescent parts of the tissue.

7.2.2. Place the slide in the slide holder and position the growth plate or other tissue of interest directly between the light path and the objective lens.

7.2.3. In order to simplify localizing the tissue, deselect the YFP and CFP channels using the tick-boxes under the **Tracks** heading of the **Channels** tab.

7.2.4. Select the T-PMT channel by clicking its name in the **Tracks** heading. Click **Live** in the **Acquisition** tab and increase the **Gain (Master)** for the T-PMT channel in order to visualize the tissue section on the screen, which is shown in greyscale.

7.2.5. Move the position of the slide using the joystick to locate the region of interest, then focus using the appropriate microscope knob.

7.2.6. Click **Stop** in the **Acquisition** tab to turn off the laser exposure.

7.3. Define the imaging parameters.

7.3.1. Use the tick-boxes in the **Channels** tab to select one of the channels and click **Live** in the **Acquisition** tab to display the image of that channel on the screen. Then, using the left-click

button on the mouse, adjust the slide-bars to set the range of emitted fluorescent signal that is collected, the laser power (sliding bar under **Lasers** heading), **Gain (Master)** and **Digital Offset** in the **Channels** tab to visualize the Confetti-labeled cells on the screen. Guideline values for all of these parameters are provided in **Supplementary file 1B-D** for RFP, YFP, and CFP. Complete this step for every channel, one by one.

NOTE: To ensure the visualized signal is not autofluorescence, a section from a Cre negative animal can be used as a negative control during this step. As T-PMT has been combined with the RFP laser (step 7.2.1) adjusting laser power for RFP will affect T-PMT signal.

### 7.3.2. Adjust the **Pinhole** size to increase the fluorescence detection if necessary.

NOTE: The higher the pinhole size, the more fluorescent signal is detected in the Z-direction. A guideline for this is the resultant Airy Unit (AU), which is automatically indicated beneath the **Pinhole** indicator, where 1 AU is optimal for imaging quality. Increasing the AU too much will impair later analysis because it becomes more difficult to distinguish individual cells in the Z-direction).

### 7.3.3. Check the setup to ensure that the recorded signals do not overlap.

7.3.3.1. Click on the tick-boxes in the **Channels** tab to select all three channels and press the **Snap** button in the **Acquisition** tab.

7.3.3.2. In the resultant image, scroll between the displayed channels by clicking the blue-highlighted boxes above each listed **Channel** in the **Dimensions** tab. Manually check for the overlap between the channels on the screen. If the signals overlap (e.g., if every red cell is also yellow), return to the beginning of step 7.3 and repeat, adjusting the parameters for the overlapping channels until they can be distinguished from each other. Ensure that for each label (i.e., RFP, YFP, or CFP), at least one clone can be visualized that contains only one of the labels.

## 7.4. Acquire image.

7.4.1. Select the **Acquisition Mode** tab to specify image quality using the **Frame Size**, **Speed**, and **Averaging Number** values.

NOTE: The typical settings for these experiments are indicated in **Supplementary file 1A**. Reducing the **Zoom** under the **Scan Area** heading within the **Acquisition** tab can increase the field of view without altering the time required for image acquisition.

7.4.2. In the **Acquisition** tab, under the **Dimensional Acquisition** heading, click the **Z-Stack** tick-box. Open the **Z-stack** tab to set the **interval** between images in the Z-direction to 2.5  $\mu\text{m}$ .

7.4.3. To set the correct locations, select only the RFP/T-PMT in the **Channels** tab and click **Live** to visualize the red clones with a visible outline of the tissue. Set the first and last slice by

clicking **Set First** and **Set Last** on the corresponding slice, adjusting the focus using the microscope knob.

7.4.4. Click the **Tile Scan** tab within the **Acquisition** tab and enter the total number of tiles in the horizontal and vertical directions in the respective boxes.

7.4.5. For image capture, select all the desired channels in the **Channels** tab, then click the **Snap** button to collect a single image, or the **Start Experiment** button to collect a **Z stack/Tile scan**.

7.5. Once the image has been acquired, click **File** then **Save As** and save the image in a file type that preserves as much information about the microscope settings as possible, to allow later review and reuse.

7.6. Perform further analysis using image analysis software.

## **REPRESENTATIVE RESULTS:**

To label chondrocytes in the epiphyseal cartilage and visualize their clonal expansion with age using tamoxifen administration on P1, Col2CreERT:Confetti mice were labeled, and their hind-limbs were collected on P27. The central section was determined by visualizing the cruciate ligament (**Figure 2A**) and clones in the proximal tibial growth plate were visualized (**Figure 2B**). These data show that individual cells that were Col2-positive on P1 and became labeled with Confetti fluorescent proteins when tamoxifen was administered, underwent clonal expansion, and subsequently remained in the growth plate until P27. The initial changes in clonal expansion from a similar developmental time point can be seen in Figure 1 of a recent publication<sup>13</sup>.

To give an example of the fluorescent signal after long-term storage (step 5.5), the section collected was stored immediately after (see **Figure 2A, B**) for more than 3 years before it was prepared and imaged (**Figure 2C, Video 1**).

To demonstrate some common problems with the protocol, a section broken during the cryosectioning step is shown in **Figure 3A**. The region of the section (between the growth plate and articular cartilage) expanded in **Figure 3B** shows that this dose of tamoxifen leads to such a high level of recombination in this area that it would preclude clonal analysis.

## **FIGURE AND TABLE LEGENDS:**

**Figure 1: Experimental overview.** Flow chart summarizing the key stages of the method.

**Figure 2: Confetti images from a Col2CreERT:Confetti mouse.** (A) Samples of Col2CreERT:Confetti mice were processed without decalcification or the long-term storage step. The central section was located using the cruciate ligament as a marker. This image was visualized using a tile scan after detecting RFP with T-PMT (Scale bar = 300  $\mu$ m). (B) Clonal columns are shown within the proximal tibia of the growth plate visible in (A), following 3D reconstruction. (C) An adjacent slide to that shown in (A) and (B) held in long-term storage for

more than 3 years was processed for imaging and 3D reconstruction. RFP, YFP, and CFP are shown in (B) and (C) (Scale bars = 50  $\mu$ m).

**Figure 3: Challenges when using the method.** (A) The white dashed lines outline a split in the section through the growth plate of Col2CreERT:Confetti mice (Scale bar = 50  $\mu$ m). The area within the white box is shown in (B) (Scale bar = 25  $\mu$ m). RFP, YFP, and CFP are shown in both panels, and the nonreflected laser light (T-PMT) channel is also shown in (A) to visualize the tissue.

**Video 1: Three-dimensional reconstruction of a growth plate from Col2CreERT: Confetti mouse.** A slide prepared from a Col2CreERT: Confetti mouse was held in long-term storage after the sectioning for more than 3 years, and then processed for imaging. Three-dimensional reconstruction was conducted automatically with image analysis software and exported as a video.

**Supplementary file 1: Typical set-up of the confocal microscope.** (A) The main controls present in the **Acquisition** tab. (B–D) The laser parameters, excitation wavelength, and the range of recorded emission wavelengths are shown for three Confetti fluorescent proteins.

## DISCUSSION:

The Confetti model was used extensively to study mineralized cartilage tissues<sup>12–14</sup> and to describe the optimized protocol. Confetti mice<sup>2</sup> with one copy or two copies of the R26R-Confetti allele can be used for breeding and experiments. Recombination in mice with one allele of the Brainbow 2.1 construct will give four potential labels: red fluorescent protein (RFP, cytoplasmic), yellow fluorescent protein (YFP, cytoplasmic), cyan fluorescent protein (CFP, membrane-localized), and green fluorescent protein (GFP, nucleus-localized), whereas recombination in the homozygous Confetti mice (i.e., containing two copies of the Brainbow 2.1 construct) will give 10 possible color outputs (RFP+RFP, RFP+YFP, RFP+CFP, RFP+GFP, YFP+YFP, YFP+CFP, YFP+GFP, CFP+CFP, CFP+GFP, GFP+GFP). However, because the DNA excision and inversion events occur in a stochastic manner, recombination to produce GFP occurs in only a small fraction of cells, as previously reported<sup>2</sup>, and seemingly differs by cell-type or Cre line used<sup>2,12,13</sup>. Hence, given the low occurrence of recombination events leading to the GFP expression, six color outputs are the most common in homozygous Confetti mice.

An important consideration when planning experiments with the Confetti mouse is to find a suitable Cre strain. First, since the Confetti allele has several loxP sites, one recombination event does not preclude a second<sup>4</sup>. This means that if a cell is labeled and divides to produce a clone of one color, a second recombination event in one of its daughter cells would generate a different color, thereby masking the true clonal expansion. Thus, noninducible Cre strains, where the enzyme is always active if the corresponding promoter is active, are not advisable. Second, in some strains the expression of Cre recombinase often comes at the expense of an endogenous protein, thereby creating a phenotype of its own (e.g., for the Prg4-GFPCreERT2 knock-in mouse strain in which mice harbor two Cre alleles<sup>15</sup>), so a single copy of the Cre allele is desirable. In all of the described experiments, a single copy of the Col2CreERT<sup>16</sup> was carried

by either the male or the female parent to generate Col2CreERT:Confetti mice, as described<sup>13</sup>. Next, the specificity of the Cre line to the cell type of interest is an important consideration. For example, Col2CreERT is chondrocyte-specific, but labels several distinct populations of chondrocytes in early postnatal cartilage<sup>17</sup>. Finally, the activity of different Cre strains can change considerably with animal age, as the endogenous activity of the promoter utilized for Cre expression changes, even in cells of the same type (e.g., *Prg4-GFPCreERT2*, which can require an increasing number of tamoxifen doses to label superficial zone cells as mice age<sup>15</sup>).

The number of labeled cells will be reflected by the amount of tamoxifen given, so it is not always desirable to give the maximum dose because distinguishing clones from each other may be difficult. Because Cre expression will vary under the regulation of different promoters as well as with age, the tamoxifen dosage will need to be adjusted to optimize labeling. It is important to note that cells are not immediately fluorescent upon triggering recombination because time is required for the recombination to occur and the resulting fluorescent proteins to accumulate sufficiently for imaging. A broad range of hours (between 24–72 h) is needed, which appears to be influenced by the Cre line, cell-type, and animal age. Since tamoxifen can be biologically active long after administration<sup>18</sup> it is always advisable to thoroughly check recombination efficiency in each model.

During the confocal microscopy step, excitation of the Confetti fluorescent proteins requires penetration of the tissue by laser light. Because different tissues have different properties, laser light might not penetrate 160 µm thick sections of all tissues. However, such thick sections can be used for growth plate cartilage, as used in **Figure 2**, **Figure 3**. Note that every microscope is different, and it is unlikely that the described settings (**Supplementary File 1**) will work perfectly without minor adjustments. One of the major challenges is to distinguish the different fluorescent proteins to ensure that there is no contamination from one channel to another. For example, the signal in the YFP channel caused by the fluorescence coming from GFP overlaps between the YFP and RFP channels. YFP and CFP channels must also be carefully checked. This can be done in several ways. First, the emission range of fluorescent light wavelengths that are recorded for each fluorescent protein can be narrowed. Second, adjusting the laser power in combination with the digital gain can optimize the signal. In this study these steps were sufficient, but they rely on a strong fluorescent signal. This means that in theory, inefficient tissue processing can reduce the activity of the fluorescent proteins, or a weak laser light source can impair channel separation.

One of the advantages of the Confetti mouse is that it can be combined with a large number of genetically mutant strains that are available so that the impact of specific genes on clonal dynamics can be studied<sup>10,11,13,14,19,20</sup>, albeit with some limitations. For example, recombination of the Confetti alleles and the gene of interest cannot be separated when using Cre loxP-based mutants combined with clonal lineage tracing. Nevertheless, such coinciding recombination events can be effective<sup>10,14,20</sup>. For example, this possibility was used to explore the increased cell number in the resting zone of mice following postnatal chondrocyte-specific ablation of TSC1 in the growth plate<sup>21</sup> and revealed the clonal relationship between these cells over time<sup>13</sup>. Additionally, the tissue-specific clonal analysis using Confetti mice can be used with non-Cre

loxP-based mutant mice<sup>11</sup>, and it is also possible to combine these approaches with pharmacological<sup>8,9,13</sup> and/or surgical models<sup>8</sup> to visualize clonal responses to other functional perturbations. Further opportunities arise when combining Confetti labeled sections with immunodetection of endogenous proteins by immunofluorescence. Such analysis allows the identification of traced cells and their colocalization with a known marker. With the fixation method described herein, it is possible to conduct immunofluorescence and still visualize the fluorescence from the Confetti fluorescent proteins<sup>12,13</sup>. However, in the papers mentioned, antigen retrieval was not required. Harsh antigen retrieval methods, such as boiling in citrate buffer, leads to a complete loss of Confetti fluorescence. Although the Confetti fluorescent proteins can then be detected by using antibodies<sup>22</sup>, a loss of clonal identity can occur.

In summary, using the Confetti model to label cells in vivo is an informative tool to analyze the fate of those cells over time. The described method provides a fast and efficient way to directly visualize fluorescent protein expression in mineralized tissues.

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#### DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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(1) Mouse breeding



(2) Confetti labeling



(3) Tissue collection



(4) Tissue fixation  
and processing



Storage at -20 °C



(5) Sectioning



Long-term  
storage at -20 °C



(6) Slide preparation  
and mounting



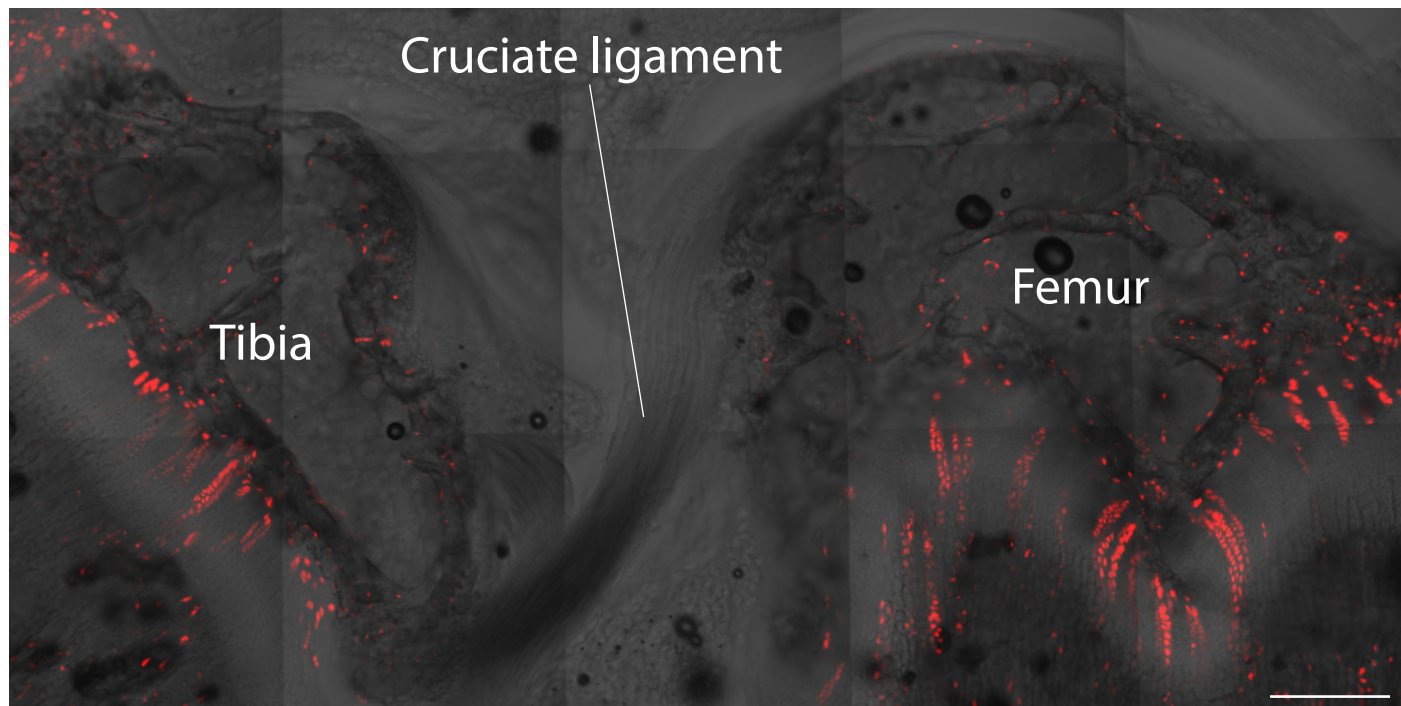
Storage at 4 °C



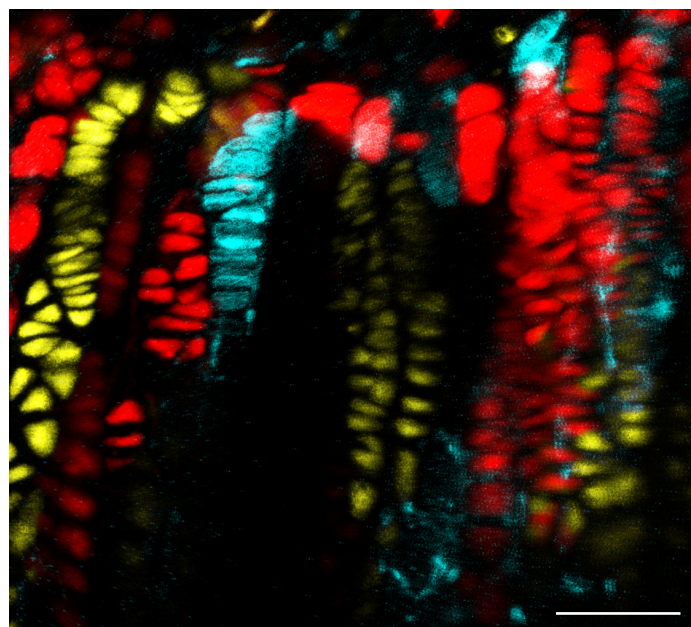
(7) Imaging by  
confocal microscopy

Figure 2

A



B



C

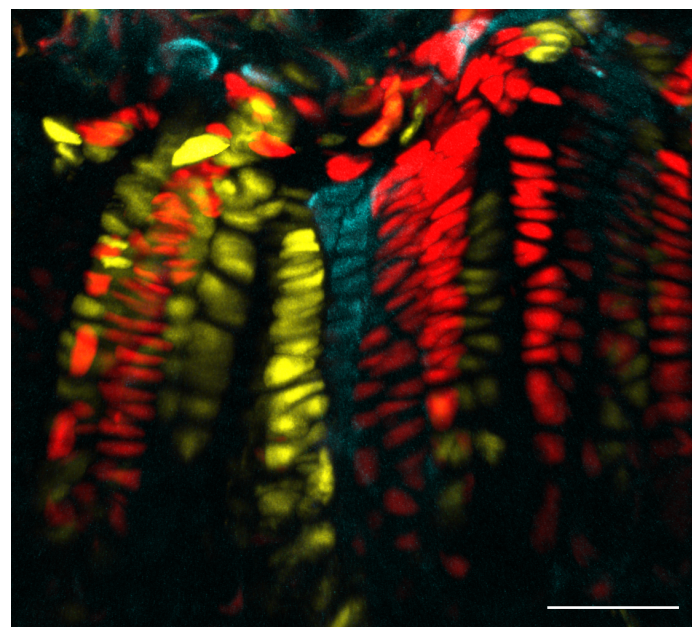
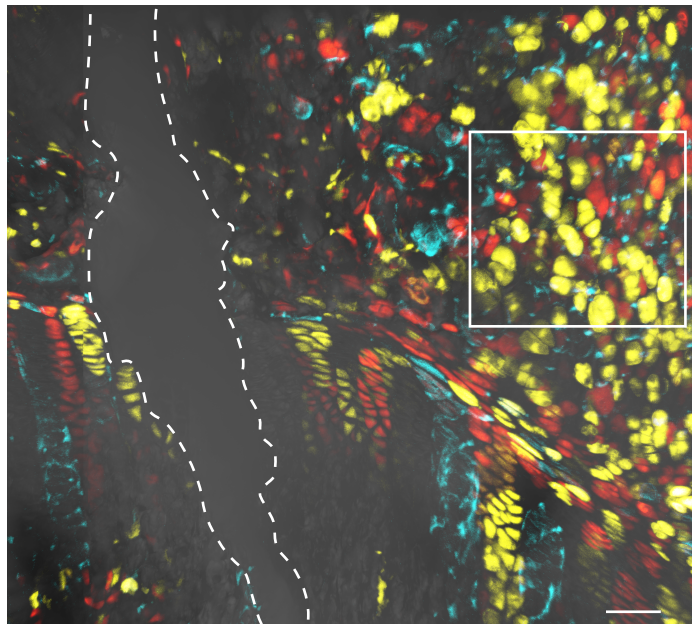
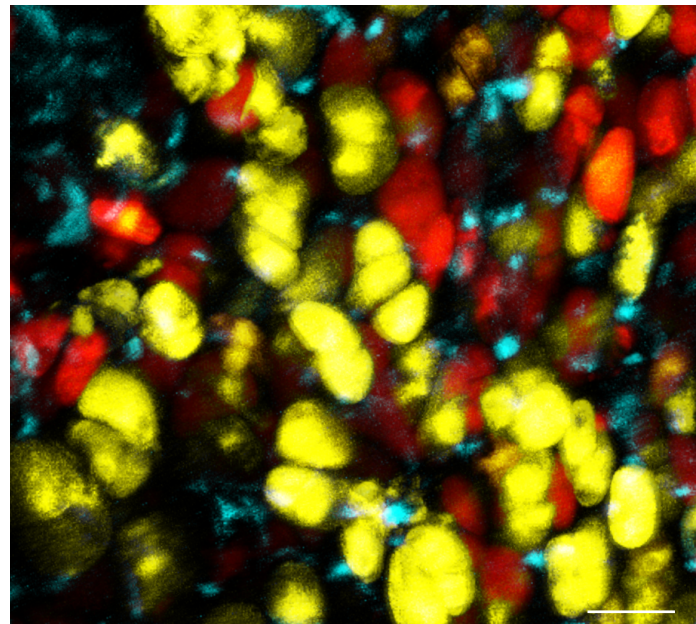


Figure 3

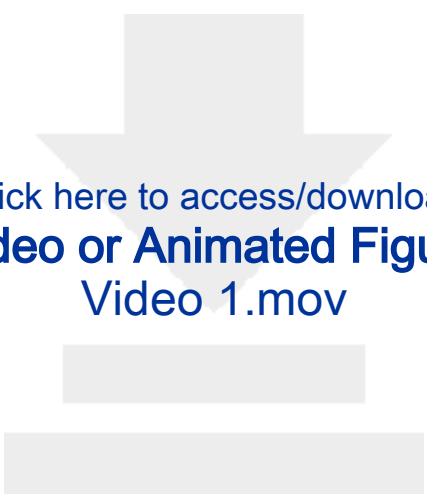
A



B



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**Video or Animated Figure**  
Video 1.mov

<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
2,2-thiodiethanol	Sigma	MKCF9328
60 mm-long cover slips	Mendel-Gläzer	220588
Confocal laser scanning microscope	Zeiss	LSM 710
Corn oil	Sigma	C8267
Cryomold (standard)	Sakura	4557
Cryostat	Thermo	Model: NX70
Disposable cryostat blades	Histolab	207500014
EDTA	Scharlan	AC0960005P
Formaldehyde concentrate	Merck	8.18708.1000
Glass bottles	Sigma	V7130
Imaris software	Oxford Instruments	N/A
Isoflurane	Zoetis	11-8162
OCT	Sakura	102094-104
Pasteur pipette	Sarstedt	86.1171
PBS	Gibco	14200075
Sodium hydroxide	Merck	1.06498.1000
Sucrose	Sigma	S0389
Superfrost UltraPlus slides	Mendel-Gläzer	J3800AMNZ
Tamoxifen	Sigma	T5648
Zen2 software	Zeiss	N/A

## Comments/Description

Specifically for use with hard tissue

Can be used for tamoxifen dilution and for tissue fixation and processing  
Image analysis software for 3D reconstruction

Freeware for Confocal laser scanning microscopy



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We have amended the protocol, representative results and legends, which previously deviated from the journal style.

We noticed that protocol steps 2 and 3 had been altered to yellow-highlights to suggest recording this part. These steps would require additional mice to be bred and euthanized and are not especially specific to the Confetti mice (as anyone trained to use laboratory animals will have been through these steps), so we have returned the yellow highlights to the previously peer-reviewed version. In general, the click-by-click software section for setting up the microscope would be more useful for new users of the Confetti mouse (protocol step 7) as it describes how to separate the channels and avoid overlapping signals, something that can be very troublesome. The instructions for every click takes up a lot of text space in order to navigate the user within the software, so adding it would far exceed the 2.75 page yellow-highlighted total; but to video such set-up would not take a prolonged period. When we originally planned this manuscript we thought it would be important to include this part but omitted it due to text length. However, it would be reasonable to think that in the final video it would take up a similar length of time to steps 2 and 3, but would be much faster to record than steps 2 and 3 (since step 7 is performed at one microscope). So if you think the video needs to be lengthened from the parts we originally suggested, please consider adding step 7 as an alternative to steps 2 and 3.

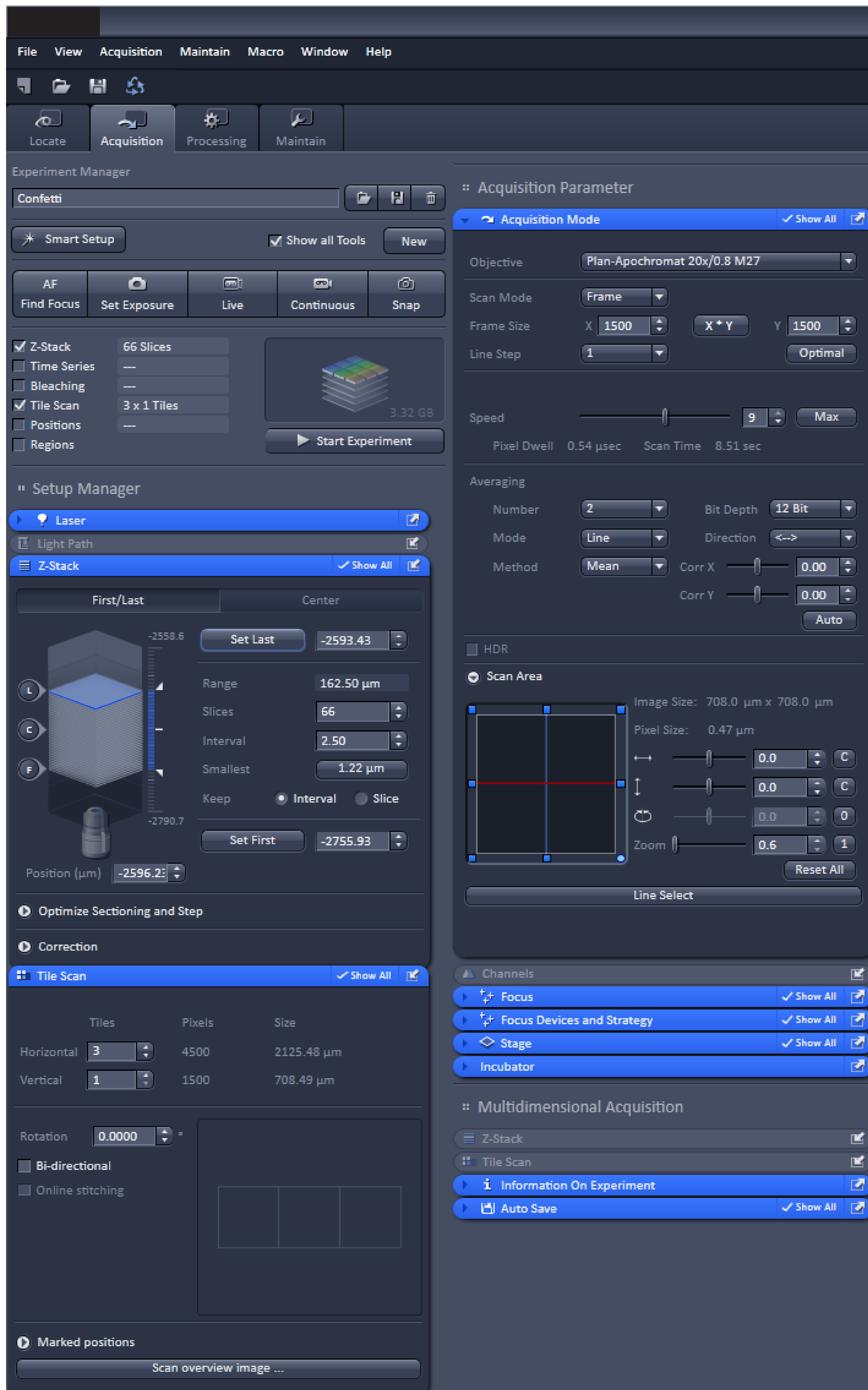
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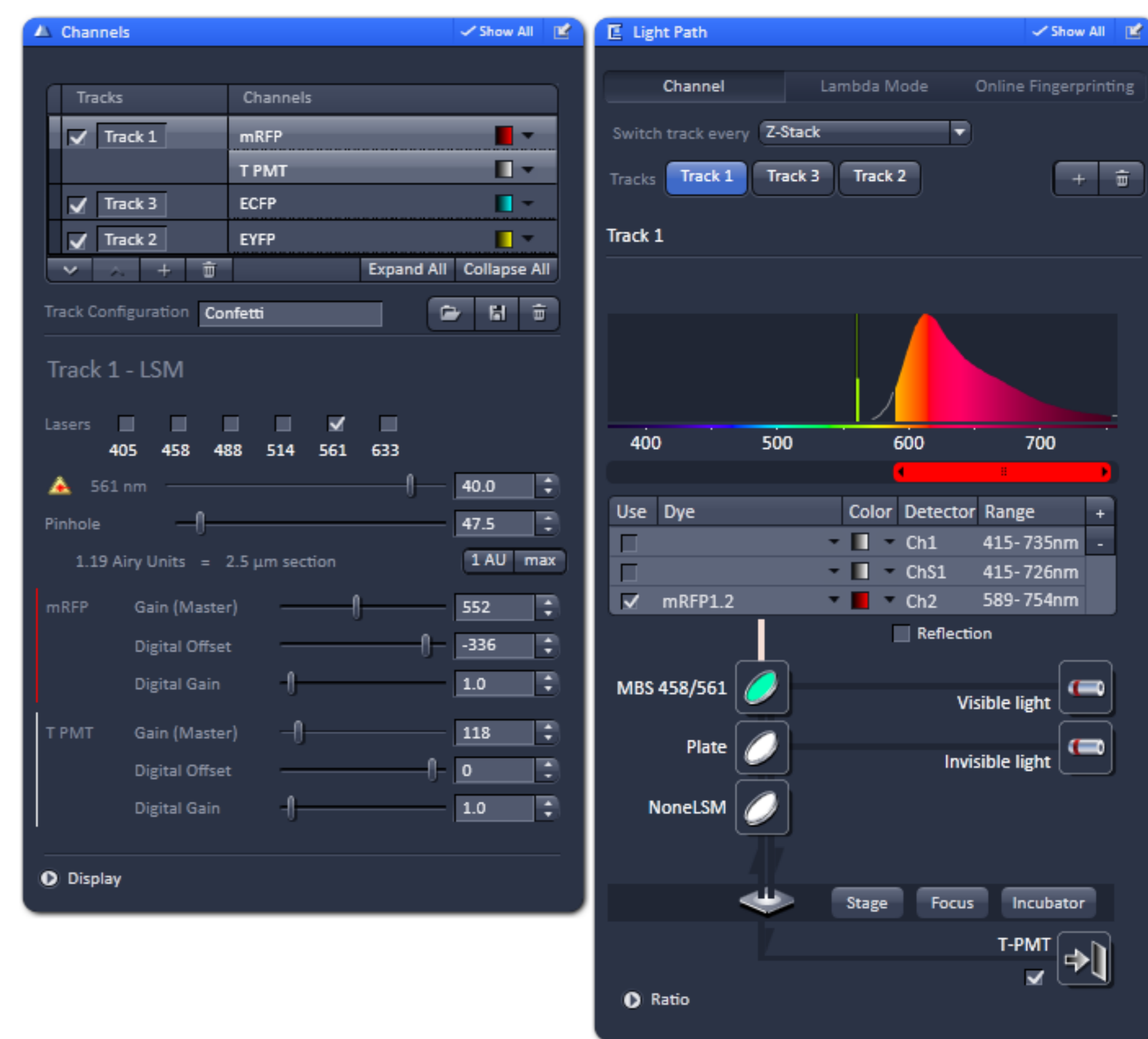
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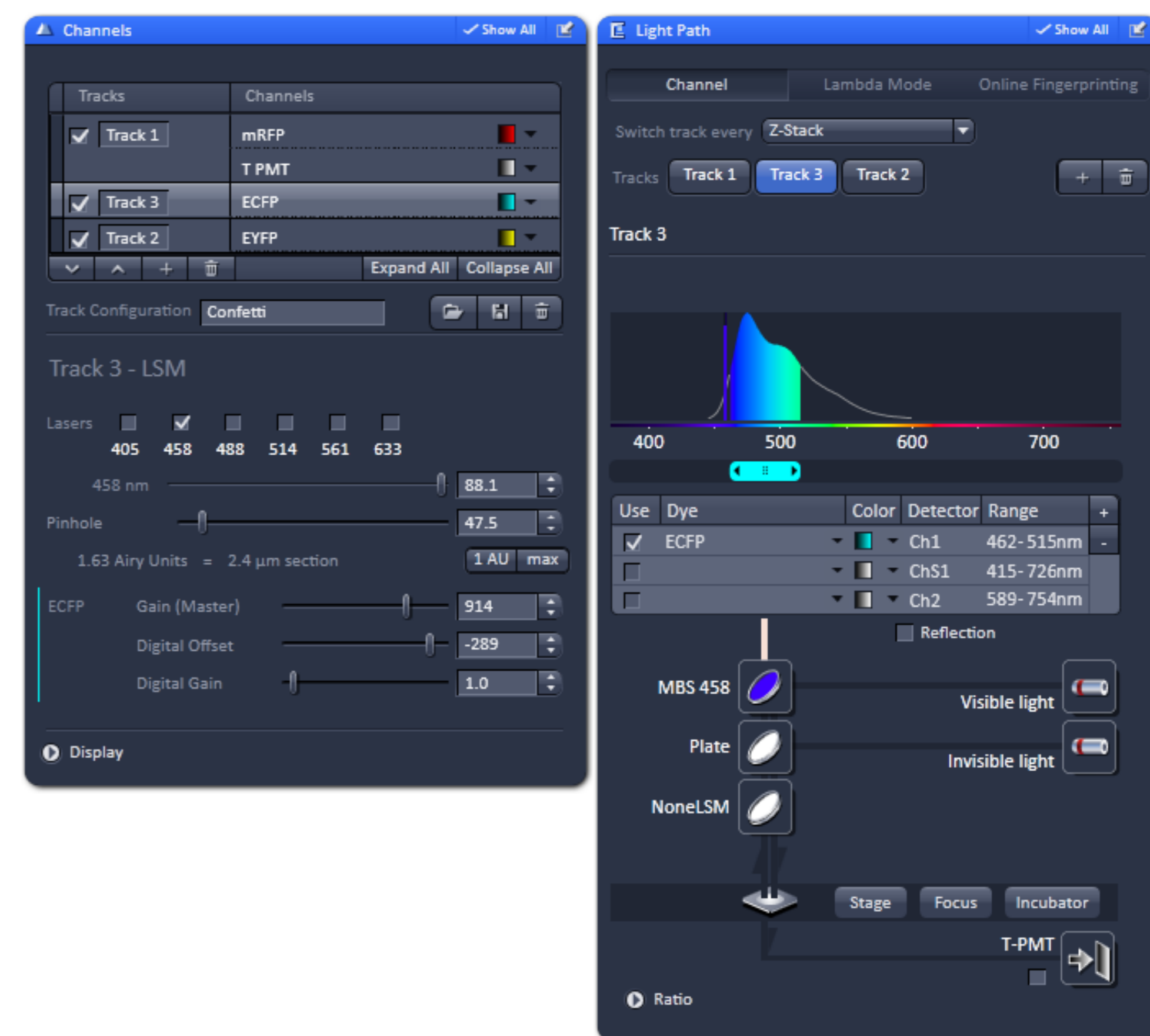
A



B



C



D

