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Isolation and Time-Lapse Imaging of Primary Mouse Embryonic Palatal Mesenchyme Cells to Analyze Collective Movement Attributes --Manuscript Draft--

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TITLE:

Isolation and Time-Lapse Imaging of Primary Mouse Embryonic Palatal Mesenchyme Cells to Analyze Collective Movement Attributes

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SUMMARY:

We present a protocol for isolation and culture of primary mouse embryonic palatal mesenchymal cells for time-lapse imaging of two-dimensional (2D) growth and wound-repair assays. We also provide the methodology for analysis of the time-lapse imaging data to determine cell-stream formation and directional motility.

ABSTRACT:

Development of the palate is a dynamic process, which involves vertical growth of bilateral palatal shelves next to the tongue followed by elevation and fusion above the tongue. Defects in this process lead to cleft palate, a common birth defect. Recent studies have shown that palatal shelf elevation involves a remodeling process that transforms the orientation of the shelf from a vertical to a horizontal one. The role of the palatal shelf mesenchymal cells in this dynamic remodeling has been difficult to study. Time-lapse-imaging-based quantitative analysis has been recently used to show that primary mouse embryonic palatal mesenchymal (MEPM) cells can self-organize into a collective movement. Quantitative analyses could identify differences in mutant MEPM cells from a mouse model with palate elevation defects. This paper describes methods to isolate and culture MEPM cells from E13.5 embryos—specifically for time-lapse imaging—and to determine various cellular attributes of collective movement, including

measures for stream formation, shape alignment, and persistence of direction. It posits that MEPM cells can serve as a proxy model for studying the role of palatal shelf mesenchyme during the dynamic process of elevation. These quantitative methods will allow investigators in the craniofacial field to assess and compare collective movement attributes in control and mutant cells, which will augment the understanding of mesenchymal remodeling during palatal shelf elevation. Furthermore, MEPM cells provide a rare mesenchymal cell model for investigation of collective cell movement in general.

INTRODUCTION:

Palate development has been studied extensively as defects in palatogenesis lead to cleft palate—a common birth defect that occurs in isolated cases or as part of hundreds of syndromes^{1,2}. The development of the embryonic palate is a dynamic process that involves movement and fusion of embryonic tissue. This process can be divided into four major steps: 1) induction of palatal shelves, 2) vertical growth of the palatal shelves next to the tongue, 3) elevation of the palatal shelves above the tongue, and 4) fusion of the palatal shelves at the midline^{1,3,4}. Over the past several decades, many mouse mutants have been identified that manifest cleft palate⁵⁻⁸. Characterization of these models have indicated defects in palatal shelf induction, proliferation, and fusion steps; however, palatal shelf elevation defects have been rare. Thus, understanding the dynamics of palatal shelf elevation is an intriguing area of research.

Careful analysis of some mouse mutants with palatal shelf elevation defects has led to the current model that posits that the very anterior region of the palatal shelf appears to flip up, while a vertical to horizontal movement or “remodeling” of the palatal shelves occurs in the middle to posterior regions of the palate^{1,3,4,9-11}. The medial edge epithelium of the palatal shelf likely initiates the signaling required for this remodeling, which is then driven by the palatal shelf mesenchyme. Recently, many researchers have identified palatal shelf elevation delay in mouse models that showed transient oral adhesions involving palatal shelves^{12,13}. The mesenchymal remodeling involves reorganization of the cells to create a bulge in the horizontal direction, while simultaneously retracting the palatal shelf in the vertical direction^{9,10,14}. Among the several mechanisms proposed to affect palatal shelf elevation and the underlying mesenchymal remodeling are cell proliferation¹⁵⁻¹⁷, chemotactic gradients¹⁸, and extracellular matrix components^{19,20}. An important question arose: is the palatal shelf elevation delay observed in *Specc1l*-deficient mice also partly due to a defect in the palatal shelf remodeling, and could this remodeling defect manifest in an intrinsic defect in behavior of primary MEPM cells²¹?

Primary MEPM cells have been used in the craniofacial field for many studies involving gene expression²²⁻²⁹, and a few involving proliferation^{30,31} and migration^{25,31,32}, but none for collective cell behavior analysis. Time-lapse imaging of MEPM cells was performed in 2D culture and wound-repair assays to show that MEPM cells displayed directional movement and formed density-dependent cell streams—attributes of collective movement²¹. Furthermore, *Specc1l* mutant cells formed narrower cell streams and showed highly variable cell migration trajectories. This lack of coordinated motility is considered to contribute to the palate elevation delay in *Specc1l* mutant embryos^{13,21}. Thus, these relatively simple assays using primary MEPM cells may serve as a proxy for studying mesenchymal remodeling during palatal shelf elevation. This paper

describes the isolation and culture of primary MEPM cells, as well as the time-lapse imaging and analysis, for the 2D and wound-repair assays.

PROTOCOL:

All experiments involving animals were carried out with a protocol approved by the KUMC Institutional Animal Care and Use Committee, in accordance with their guidelines and regulations (Protocol Number: 2018-2447).

1. Harvest E13.5 embryos

1.1. Euthanize pregnant female mice using a CO₂ inhalation chamber or by a procedure approved by the Institutional Animal Care and Use Committee. Immediately proceed to dissection.

1.2. Expose the inferior half of the abdominal cavity by removing the skin and peritoneum. Excise both horns of the uterus, which contain the E13.5 embryos.

1.3. Briefly place the uterus in prewarmed 37 °C sterile phosphate-buffered saline (PBS) to rinse off excess blood, hair, or other debris. Place the uterus in a sterile 10 cm dish filled with sterile PBS.

1.4. Using small scissors, cut through the uterine wall along the length of the uterus to expose each embryo, still in its yolk sac. Remove the yolk sac surrounding the embryo, but save it for genotyping, if needed. As the embryos are removed, place each embryo in its own well of a sterile 12-well plate filled with sterile PBS.

2. Dissection of palatal shelves from embryos (Figure 1)

NOTE: Sterilize the stainless steel dissection instruments (see the **Table of Materials**) after processing each embryo by placing the instruments first in a beaker of 100% ethyl alcohol (EtOH), then in an instrument sterilizer at 350 °C for 10 s, and then cooling them in a second beaker of 100% EtOH.

2.1. Using a sterilized perforated spoon, place the embryo in a new sterile 10 cm dish filled with sterile MEPM culture medium consisting of Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (4 mM L-Glu), and the antibiotics—penicillin and streptomycin (50 units/mL).

2.2. Decapitate the embryo right below the jaw line using sterile scissors (**Figure 1A**, red dotted line). Remove the lower jaw by inserting one point of a sterilized fine #5 forceps into the mouth, keeping it just inside the cheek. Push the point of the inserted forceps through until it exits out the back of skull.

2.3. Orient the forceps, along the yellow line in **Figure 1B**, so that the other side of the forceps (which is still outside of the embryo) is hovering just over the ear canal, then pinch the forceps shut to cut the tissue. If necessary, run another fine forceps along the seam of the now closed forceps to cut through any tissue that was not completely severed by the pinch.

2.4. Repeat the previous step for the other side of the embryo head. Continue the pinch-cut procedure to fully remove the lower jaw, tongue, and inferior portion of the skull and expose the palatal shelves.

2.5. Remove the cranium of the skull by cutting just above the eyes, as shown in **Figure 1C** (green line). Do this by placing the head on its left or right side and positioning the points of a small sterile stainless steel scissors in front of and behind the skull just above the embryo's eye level. Cut off the top of the skull with one fast snip of the scissors, creating a flat surface that will be important for stability in later steps and that should look like **Figure 1D** when viewed from the side.

2.6. Place the remaining part of the head upside down, with the superior aspect of the head (cranium removed) resting flat on the bottom of the dish, which will provide a stable surface for palatal shelf removal. Take a moment to identify the palatal shelves, which are now exposed and facing up and will appear as two raised ridges on either side of a central groove in the anterior half of the head (**Figure 1E**).

2.7. Pin the remaining portion of the head to the dish to immobilize it while the shelves are removed. Do this by inserting one point of a fine forceps through the tissue near the nasal region of head, anterior to the palatal shelves, and insert the other point of the forceps through the base of the skull, posterior to the palatal shelves. Hold these in place while performing the excision of the palate shelves.

2.7.1. Immobilizing the head with one hand, pick any one of the two shelves to remove first, and insert both points of a second fine forceps into the tissue at the base of the lateral surface of the shelf, and pinch to cut the tissue (**Figure 1F**). Repeat this along the base of the medial surface of the shelf and then at both the anterior and posterior ends of the shelf to detach the shelf from its attachment to the head.

2.8. Gently lift the shelf, making additional pinches, as needed, to completely free the shelf from the surrounding tissue.

2.9. Repeat the previous two steps to remove the second palatal shelf.

2.10. With the palatal shelves now freed from the surrounding tissue and placed in PBS (**Figure G**), use a sterile plastic bulb transfer-pipette to draw up the shelves in the pipette, and transfer them into a sterile 1.5mL microcentrifuge tube along with approximately 500 μ L of sterile PBS. Keep the tubes containing palatal shelves on ice as the rest of the litter is processed in the same fashion.

NOTE: Alternatively, shelves can be placed in 1.5mL microcentrifuge tube containing prewarmed trypsin (0.25%) immediately after dissection (in lieu of placing them on ice). Samples will be fresher, but care must be taken to time all the proceeding steps for each individual sample as opposed to treating the samples collectively.

3. Culture of MEPM cells

NOTE: Under the conditions described here, the palate epithelial cells do not survive the first passage, resulting in a pure palate mesenchymal cell culture. Use sterile technique to perform all steps in a tissue culture hood.

3.1. Aspirate and discard the PBS from the 1.5 mL tube, taking care not to discard the shelves in the process. Immediately add 200 μ L of prewarmed (37 $^{\circ}$ C) trypsin (0.25%) to each tube that contains palatal shelves. Briefly pipet the shelves up and down in the trypsin using a 1000 μ L pipette tip to accelerate the trypsinization.

3.2. Incubate the tubes for 5 min at 37 $^{\circ}$ C, then pipet each sample up and down again to help break up the tissue. Incubate the tubes for another 5 min at 37 $^{\circ}$ C, and pipet up and down once more to complete the dissociation of the tissue.

NOTE: The shelves must be completely or nearly completely dissociated and suspended in the trypsin with no visible chunks of tissue remaining.

3.3. Add 800 μ L of MEPM culture medium (step 2.1) to each 1.5 mL tube. Centrifuge the 1.5 mL tube at $200 \times g$ for 5 min to pellet the cells. Remove the supernatant, and resuspend the cell pellet in 1 mL of MEPM culture medium.

3.4. Plate the MEPM cells into a 6-well tissue culture-treated plate containing MEPM culture medium. Allow the cells to adhere to the plastic surface for 12 h at 37 $^{\circ}$ C in a sterile incubator with 5% CO₂.

NOTE: After overnight incubation, the vast majority (~90%) of cells will attach. At this point, the adhered cells will look fairly homogeneous, with a triangular or slightly elongated shape.

3.5. Change the medium every day by gently aspirating the old medium and immediately replacing it with 1 mL of warm sterile PBS without calcium or magnesium for ~1 min. Aspirate the PBS, and replace with 3 mL of prewarmed MEPM culture medium.

3.6. Passage the cells once they become 100% confluent.

NOTE: MEPM cells should proliferate by doubling in number almost daily.

3.6.1. To passage the cells, gently aspirate the old medium, and immediately replace it with warm sterile PBS without calcium or magnesium for ~1 min. Aspirate the PBS, and replace it with 0.5 mL of prewarmed 0.25% trypsin.

3.6.2. Incubate at 37 °C for ~5 min, or until the cells detach from the surface of the dish when gently rocked back and forth by hand. Once the cells have detached, immediately add 5 mL of prewarmed MEPM culture medium to the trypsinized cells.

3.6.3. Using a sterile 10 mL serological pipet, gently collect the cells in a 15 mL conical tube, and centrifuge the tube at $200 \times g$ for 5 min to pellet the cells. Aspirate the trypsin and medium, and resuspend the cells in 3 mL of prewarmed MEPM culture medium. Gently pipet 1 mL of cells into a single well of a 6-well dish, and add 2 mL of MEPM culture medium to bring the total volume to 3 mL.

NOTE: This constitutes a 1:3 split of cells. MEPMs may be passaged up to three times. The seeding density of MEPMs is somewhat flexible, and the number of cells present varies depending on the culturing vessel. However, MEPMs do not properly proliferate when split too sparsely and should be at least 20–25% confluent in their new dish once they adhere.

4. Cryopreservation of MEPM cells

4.1. Once trypsinized MEPM cells are pelleted, resuspend the cells in MEPM culture medium to obtain a concentration of $\sim 1 \times 10^6$ cells/mL. Pipet the cells into cryovials, and add a final concentration of 5% dimethylsulfoxide in the cell stock. Cap the cryovial, briefly mix by inverting, and immediately place the vials in a freezing container that cools at a rate of 1 °C/min. per min.

4.2. Place the cooler in a -80 °C freezer overnight. On the next day, move the cryovials to a liquid nitrogen tank for long-term storage.

4.3. Thawing cryopreserved MEPM cells

4.3.1. Remove the cryovials from the liquid nitrogen tank, and thaw at room temperature until the contents begin to become liquid. Empty the contents into a 15 mL conical tube containing 9 mL of prewarmed MEPM culture medium.

4.3.2. Centrifuge the tube at $200 \times g$ to pellet the cells. Resuspend the cells in 1 mL of MEPM culture medium, and pipet the cells into a single well of 6-well plate. Add 2 mL of warm MEPM culture medium to bring the total volume to 3 mL.

4.3.3. Culture the cells at 37 °C in a sterile incubator with 5% CO₂. Change the medium daily.

5. Live-imaging of MEPM cells—2D collective migration assay (Figure 2)

5.1. Prepare a plate to use for live imaging.

5.1.1. Use small surgical scissors or a sharp scalpel to shorten a sterile 2-well silicone insert to a height of ~1 mm. Prepare an insert for each sample being used.

5.1.2. Using sterile forceps, place the shortened sterile 2-well silicone insert in the center of a well of a 6-well plate. Press down along all edges to ensure it is fully adhered.

5.2. Thaw cryopreserved cells by following the steps in section 4.3 of this protocol. Count MEPM cells, and seed 300 cells/mm² of the shortened silicone inserts in a total volume of 40–50 µL MEPM culture medium per well. Culture the cells overnight at 37 °C in a sterile incubator with 5% CO₂.

5.3. On the next day, prepare for live time-lapse imaging.

5.3.1. Use a phase contrast microscope with an on-stage incubator and automatic imaging capability. Add water to the onstage incubator reservoir to reduce evaporation of the culture medium; set the temperature to 37 °C and CO₂ to 5%. Allow ~30 min for the humidity to build up before placing the 6-well dish in the onstage incubator.

5.4. Use settings equivalent to the following for time-lapse imaging.

5.4.1. Select the 4x objective to have large fields of view and phase contrast filter.

NOTE: Autofocus, Auto find sample, z-stack, and auto-lighting are not usually necessary.

5.4.2. Select two microscopic fields per well to capture the lumen of the shortened silicone inserts. Ensure all imaging positions have the correct focus.

NOTE: Image planes can be adjusted during imaging, but such adjustment is not usually necessary.

5.4.3. Select the desired image output file-type, then select or de-select post-imaging options, such as automatic video creation and watermarks, as desired. If applicable, select phase contrast as imaging mode.

NOTE: Using watermarks may impede subsequent image processing steps.

5.4.4. Set the duration of the recording to 72 h. Set the program to capture images every 10 min.

NOTE: Usually only 48 h of imaging is required, but imaging can be stopped at any point before the 72 h mark without losing images that have already been taken.

5.4.5. Make sure the environmental chamber is operational as required in 5.3.1. Save these settings (**the routine**) and begin imaging.

307
308 5.5. Continue imaging until 72 h (or the specified time).
309

310 **6. Live-imaging of MEPM Cells in a wound-repair assay (Figure 3)** 311

312 6.1. Prepare a plate to use for live-imaging. Using sterile forceps, place a sterile 2-well silicone
313 insert in the center of a well of a 6-well plate, and press down along all edges to ensure it is fully
314 adhered. Prepare one 2-well insert for every sample being used.
315

316 6.2. Thaw cryopreserved cells by following the steps in section 4.3 of this protocol. Count MEPM
317 cells, and if necessary, concentrate the cells to at least 350 cells/ μL .
318

319 6.3. Seed 1400 cells/ mm^2 of the silicone inserts in a volume of 100 μL of MEPM culture medium
320 per well. Culture the cells for 48 h at 37 °C in a sterile incubator with 5% CO_2 , and change the
321 medium every day.
322

323 6.4. After 48 h of seeding the cells, prepare a microscope for live time-lapse imaging as described
324 in sections 5.3 and 5.4. Immediately prior to placing the cells in the onstage incubator, add 3 mL
325 of prewarmed MEPM culture medium to the well (but outside of the inserts), and then carefully
326 remove the silicone inserts.
327

328 NOTE: The wall separating the 2 chambers leaves a gap that is the “wound”.
329

330 6.5. Start time-lapse imaging as described in 5.4, with the following differences:
331

332 6.5.1. Use a higher magnification (e.g., 10x) objective. To capture wound closure, select 5 fields
333 of view along each wound, so that the wound is parallel with the vertical axis of the image.
334

335 6.6. Stop imaging after 72 h or when the wounds have fully closed.
336

337 **7. Computational analysis of time-lapse image sequences** 338

339 NOTE: Perform the following procedures on a computer equipped with standard computational
340 tools, such as the python interpreter, C compiler, and a shell (see the **Table of Materials**).
341

342 **7.1. Confluency analysis** 343

344 NOTE: This procedure can be used to estimate cell proliferation within a sparse culture or to
345 quantify wound closure experiments. To detect areas occupied by cells, a segmentation threshold
346 is applied to the local standard deviation of image brightness. The code has been described
347 previously by Wu et al.³³ and Neufeld et al.³⁴ and is available at
348 <http://github.com/aczirok/cellconfluency>.
349

7.1.1. Determine the segmentation threshold for the images. As an example, to see the segmentation with a threshold 4, issue the command

```
segment.py -i inout-image.jpg -S 4 -test output.jpg
```

and then check the output (**output.jpg**).

NOTE: If the threshold is too low, background areas in the micrograph are classified as cell-covered. In contrast, if the threshold is too high, cell-covered areas are not classified as such. The optimal threshold value keeps both errors at a minimum.

7.1.2. Use the provided area.sh script to calculate confluency values for a sequence of images as

```
area.sh -S 4 img_001.jpg img_002.jpg .... > confluency.dat
```

NOTE: The discrimination threshold value 4 is verified in step 1, and the results are stored in the file **confluency.dat**. By sorting the images into appropriate folders, the list of image file names can be replaced by **wildcard** notation:

```
area.sh -S4 *.jpg > confluency.dat
```

7.1.3. For wound-closure experiments, transform the confluency data $A(t)$ —the size of cell-covered area expressed as a percentage as a function of time—into wound-edge propagation speed V as

$$V = \frac{w}{2} \cdot \frac{dA}{dt},$$

where w denotes the width of the field and dA/dt is the time derivative of $A(t)$, i.e., the expansion rate of the cell-covered area.

7.2. Cell motility map

7.2.1. Execute a particle image velocimetry (PIV) algorithm to characterize cell motility, and extract the extent of local movement “optical flow” between image pairs, but not to identify individual cells.

NOTE: Here, an initial window size of 50 μm is used, as described in detail by Zamir et al.³⁵ and Czirok et al.³⁶, with an initial window size of 50 μm . The PIV analysis yields a velocity field $v(x,t)$ for each image frame t and location (within the image) x .

7.2.2. Extract the average speed of cell motility from $v(x,t)$ as a spatial average calculated over the cell occupied area, as determined in section 7.1.

7.3. Manual cell tracking

NOTE: While the PIV analysis provides an automatic assessment of cell motility, to focus on the behavior of individual cells often requires manual tracking. While several tools provide this functionality, it is very helpful if the manually positioned markers can be modified after their initial placement, and if tracking can be performed both forward and backward in time.

7.3.1. Perform cell tracking with a custom-developed python tool (http://github.com/donnagreta/cm_track), which also provides basic editor functions such as deleting trajectory segments.

NOTE: This manual tracking tool yields the positions $P(i,t)$ of cell i at time t in a text file, and invoked as

cm_track.py -i images/ -o track.dat

where the time-lapse images are in the folder images/ and the position data is collected in the file **track.dat** (Figure 4).

[PLACE FIGURE 4 HERE]

7.3.2. Overlay trajectories on images using a second tool, invoked as

visdat.py -d track.dat -i images/ -o overlay/ -l999 -r3 -C2

7.3.3. Collect the images with the trajectories overlaid in the folder overlay.

NOTE: In this example, cell position data are stored in the file **track.dat**, while the time-lapse image sequence is within the folder images. The rest of the parameters control the maximal length of the trajectories drawn (-l), the size of the symbols (-r), and the color scheme (-C) used.

7.4. Analysis of individual cell trajectories

7.4.1. Characterize trajectories by the total path length

$$P(i,t) = \sum t' = O^t |P(i,t' + 1) - P(i,t')|,$$

and net displacement toward the wound

$$D(i,t) = |X(i,t) - X(i,0)|,$$

where X denotes the projection of P in the direction perpendicular to the wound: the x coordinate of the positions when the wound is parallel to the y axis.

7.4.2. Calculate the guidance efficiency as $\frac{D(i,t)}{P(i,t)}$ for each cell i and time point t ³⁷. Characterize cultures by the population average of these single cell measures, evaluated at a suitable time point t .

7.5. Collective streaming motion of cells

7.5.1. Characterize the local spatial correlations of cell movements by the average velocity of other cells that are in the vicinity of a moving cell, as described previously^{38,39}.

NOTE: The computational code is available at <https://github.com/aczirok/flowfield> .

7.5.1.1. Align a reference system with directions front, rear, left, and right to each vector $v(x,t)$ (**Figure 5A**). Assign each of the surrounding cell or PIV velocity vector to the appropriate spatial cell of the reference system (**Figure 5B**). Repeat the procedure as each vector serves as the origin of the reference systems (**Figure 5C,D**) so that a given velocity vector is assigned to multiple bins.

NOTE: A data point could be in front of a vector, and to left of another one.

7.5.1.2. Rotate the reference systems into a common orientation (**Figure 5C,F**) and pool them (**Figure 5G**).

NOTE: The average of each bin of the pooled velocity data is a velocity vector (U) that is indicative of spatial correlation: the average is a measure of a shared velocity component (**Figure 5H**).

7.5.1.3. Fit the $U(x)$ flow fields with an exponential function $U(x) = a \cdot e^{-\frac{x}{x_0}} + U_0$, where a , x_0 and U_0 are fitting parameters. Out of the three fitting parameters, focus on x_0 , the correlation length (**Figure 5I,J**), which is the characteristic distance where local velocity-velocity correlations disappear.

[PLACE FIGURE 5 HERE]

REPRESENTATIVE RESULTS:

The dissection of palatal shelves is illustrated in **Figure 1**. The sequence of incisions is designed to minimize slippage of the tissue. Following the removal of the head (**Figure 1A,B**), the lower jaw is removed (**Figure 1B,C**). The incision of the upper part of the head (**Figure 1C,D**) is done to stabilize the tissue when placed upside down (**Figure 1E**) to visualize (**Figure 1E, dotted lines**), pinch (**Figure 1F**), and excise (**Figure 1G**) the palatal shelves.

The excised palatal shelf pair from a single embryo are trypsinized and cultured in a 35 mm dish or in a well of a 6-well dish. Larger dishes are not preferred as the cell density is too low for optimal growth. Upon confluence, the cells from each well are trypsinized and passaged into three 35-mm-equivalent wells (passage #1). Confluent cells from passage #1 can then be frozen down into aliquots of 1×10^6 cells/mL. Frozen aliquots are subsequently brought up in a 35-mm-equivalent dish and grown to confluence. The cells are then trypsinized (passage #2) and seeded according to the experiment. Creating and using frozen aliquots helped to normalize conditions, especially with respect to cell density. The use of fresh MEPM cells resulted in more variability in final cell density in experiments, which is believed to be due to a variable proportion of viable or sub-viable cells in fresh cultures. In addition, the number of MEPM cell passages were strictly limited to two (listed above) for these experiments.

Considering that a) cell density was critical, b) MEPM cells from a single embryo were limited, and c) imaging optics were better in a large dish, 2-well silicone inserts were used (**Figure 2** and **Figure 3**). For wound-repair assays, MEPM cells were grown in the 2-well silicone inserts until high confluence, then the inserts were removed, and the wound imaged until closure (**Figure 3**).

However, for 2D culture, the MEPM cells needed imaging as they grew, so the 2-well silicone inserts were simply trimmed to 1/3rd of their height to allow for clear imaging (**Figure 2C**). Small 3D printed rings⁴⁰ in 35 mm dishes were also used for 2D motility analysis (**Figure 2D**).

MEPM trajectories (**Figure 4E**) are persistent: the direction of cell motility is maintained for several hours. The mean displacement vs. time analysis (**Figure 4F**) indicates the persistence in the form of displacement being proportional with elapsed time. The typical speed of MEPM cells on tissue culture plastic surface, 10 $\mu\text{m}/\text{h}$, can be also used for quality control of the cell culture. Flow analysis of the motility data reveals that the co-moving clusters of MEPM cells are $\sim 300 \mu\text{m}$ in size (**Figure 5I,J**). The representative results also indicate a profound motility difference between wild-type and mutant MEPM cells. In addition to wildtype and mutant comparison, both 2D and wound-repair assays can be combined with various biochemical treatments. For example, PI3K-AKT pathway activators have been used with MEPM cells, as described previously²¹.

FIGURE AND TABLE LEGENDS:

Figure 1: Dissection of embryonic palatal shelves to isolate mesenchymal cells. (A) An E13.5 mouse embryo head is removed by cutting along the neck (red line). (B) Next, the lower jaw and tongue are removed with incisions along the oral cavity, between the upper and lower jaws (yellow line). (C) To stabilize the tissue for palatal shelf removal, the top of the head is excised (green line). (D) The resulting dissected upper jaw region is placed (E) upside down to visualize the palatal shelves (black dotted lines). (F) The excision of individual palatal shelves is depicted schematically, where individual protruding shelves (yellow) are pinched off from the maxilla (blue). (G) Excised pair of palatal shelves form a single embryo, which can then be trypsinized and cultured in a 35 mm dish or in a 6-well plate.

Figure 2: Experimental setup of 2D MEPM cell culture. (A) Thaw a frozen aliquot of MEPM cells, and (B) culture the cells in a 35 mm dish or a 6-well plate. When confluent, trypsinize the cells and seed them as described in the protocol in a 35 mm dish either with (C) 2-well silicone inserts that have been trimmed or (D) with 3D-printed rings. A small culture space minimizes the need for large numbers of MEPM cells. The low profile of the trimmed silicone inserts or the 3D-printed rings allows for direct imaging without a halo effect. Time-lapse imaging can continue until the desired cell density is achieved, which can be up to 72 h. Representative images are shown at (E) 0 h, (F) 24 h, and (G) 45 h timepoints. The images were taken using a 4x objective. The scalebars for E, F, G = 300 μm . Abbreviations: 2D = two-dimensional; MEPM = mouse embryonic palatal mesenchyme; 3D = three-dimensional.

Figure 3: Experimental setup of wound-closure assay using MEPM cells. (A) Thaw a frozen aliquot of MEPM cells, and (B) culture cells in a 35 mm dish or a 6-well plate. When confluent, trypsinize the cells and (C) seed them in the 2-well silicone inserts in a 35 mm dish. Culture the cells in the insert until the desired confluence (~ 48 h), then remove the silicone insert and image. Representative images are shown (D) immediately after removal of the insert, (E) after 24 h, and (F) after 40 h. Wound closure takes around 36 h. The images were taken using a 10x objective. The scalebar represents 300 μm . Abbreviations: MEPM = mouse embryonic palatal mesenchyme.

Figure 4: Analysis of individual cell trajectories. (A) Phase-contrast time-lapse micrographs are subjected to (B, C) a manual tracking procedure, which marks cells (green dots). (D) Cell positions (x,y) are stored for each cell distinguished by its ID and for each frame f. (E) Trajectories can be overlaid on the micrographs and color-coded to indicate temporal information. As an example, in each trajectory, a blue to red color palette indicates progressively later trajectory segments, with red and blue marking the initial and final cell locations, respectively. (F) Various statistical properties of trajectories, such as the mean square displacement, can be extracted and used to characterize the motility of various cell populations, which in this example include wildtype (wt, blue), and knockdown (kd, red) MEPM cells. The scalebars represent 100 μm .

Figure 5: Characterization of stream formation of cultured cells. (A,D) Phase-contrast time-lapse images from **Figure 4A** are used to identify cell movements. For each moving cell, a frame of reference (blue) and spatial bins (white) were co-aligned to categorize adjacent cells as being in the front, rear, left, or right. (B,E) The velocity of adjacent cells (black vectors) was related to the same frame of reference (C,F). This procedure was repeated for each cell and time-point. (G) After pooling this local information, each bin will contain multiple velocity vectors (gray), which can be averaged to determine the average co-moving velocity (magenta arrows) at various locations relative to an average motile cell. (H) The average velocity map thus characterizes the typical cell velocities at various locations relative to a moving cell. (I,J) Finally, this field was sampled along the front-rear (parallel) axis and also along the left-right (perpendicular) axis.

DISCUSSION:

Palatal shelf elevation constitutes a vertical to horizontal remodeling event^{1,3,4,9,11}. It is postulated that this remodeling process requires palatal shelf mesenchymal cells to behave coordinately. The analyses with wildtype MEPM cells show that this cell behavior is intrinsic and can be quantitated²¹. Thus, these assays can be used to uncover primary palatal shelf elevation defects in new and existing mouse models of cleft palate. The methods outlined in sections 1 and 2 should allow investigators to isolate, culture, and freeze aliquots of primary MEPM cells. These cells can then be used for a wide variety of applications, including the 2D culture and wound-repair assays described here. The 2D culture, when combined with time-lapse imaging, presents a simple method to determine basic cell attributes over a range of cell densities. Presented here is a method to assess cell alignment and cell stream formation, which are attributes of collective cell movement (**Figure 4**). Wound-closure assays are commonly used to assess cell migration⁴¹⁻⁴³. The cell-free region of the “wound” provides a cue for directional movement of cells at the periphery. Time-lapse imaging of this process allowed the determination and assessment of cell trajectories during migration. These cell trajectories, in turn, determine cell migration speed and directionality (**Figure 5**).

It is important to first optimize conditions for culture and assays using only wildtype MEPM cells. After the palatal shelves have been successfully trypsinized and the resulting single-cell suspension plated overnight, the vast majority (~90%) of cells will readily attach to the growth-surface of the dish. If the cells do not readily adhere, then there may be something wrong with the culture conditions. Initially, the adhered cells will look fairly homogeneous, with a triangular or slightly elongated shape, but as they grow to a high density, they become more spindle-shaped

and elongated (**Figure 2**). If the cells become dramatically large in size or multinucleated, they should not be used for experiments. This optimization will also establish basic wildtype parameters for successful growth (time to confluence) and wound-repair (time to closure). The cells should proliferate by doubling in number almost daily and in time-lapse images, show motility of ~5–10 $\mu\text{m}/\text{h}$. Similarly, if in any subsequent experiment involving a wildtype–mutant comparison, these basic parameters are not met by wildtype cells, the experiment may need to be excluded from analysis. For example, wildtype MEPM cells take 36–40 h to completely close the wound using the 2-well silicone inserts. If in an experiment, wildtype cells take significantly longer than 40 h to close the wound, the entire experiment would be suspect. Poor performance from MEPM cells may be due to 1) poor frozen aliquot quality, 2) poor revival, or 3) excessive differentiation. Differentiated or senescent cells can be detected visually as they grow very large and do not divide. A culture with a large number of such cells should be avoided. In general, there should be no abnormal cells in the field of view of a 10x objective (**Figure 3**); a cell or two outside the field of view should not materially affect the analysis. Restricting analysis to only two cell passages (above) greatly helps maintain the quality of MEPM cells.

For both the 2D culture and wound-repair assays using primary MEPM cells, the key requirement for success was a high initial cell density. This requirement was first determined during optimization of wound-repair assay in which cells were seeded at >1400 cells/ mm^2 . The density of cells migrating into the wound was then used as the seeding density in 2D culture assays. A seeding density of ~ 300 cells/ mm^2 formed cell streams, while still allowing for automated analysis of cell trajectories. Densities higher than 300 cells/ mm^2 tend to form more vivid streams that can be visualized by eye, but make individual cell tracking difficult. When comparing primary MEPM cells from wildtype and mutant embryos, although wound-closure delay can be assessed without computational analysis, stream formation and directionality differences may not be discernable by eye. If the cell density is too high or image quality is poor/blurred, for example due to loss of focus, automated cell tracking may be difficult. In such a case, it is possible to use manual cell tracking to determine cell trajectories in wound-repair assays using ImageJ. A very low concentration of Hoechst nuclear stain (3 $\mu\text{L}/\text{mL}$ of 20 mM solution in MEPM culture medium) can also be used to facilitate cell tracking; however, fluorescent laser toxicity can be an issue with extended use.

For manual tracking, it was easier to track cells in reverse from the end of wound-closure backwards as far as possible until high cell density obscured further tracking. Even partial cell trajectories, when combined for a cell population, were informative. In contrast to wound-repair assays, automated cell tracking is required for 2D cell culture analysis, which is one reason why intermediate cell densities were chosen. Lastly, sensitive primary MEPM-based analyses can be used to identify compounds and pathways that may affect palate elevation. Previous studies indicated that activation of the PI3K-AKT pathway improved both cell speed and directionality of *Specc1l* mutant MEPM cells²¹. Other MEPM studies have also used various growth factor or drug treatments to stimulate downstream signaling cascades or to assess proliferation^{22,29,30,44,45}. Thus, MEPM-based analyses offer a quick method to identify many more positive or negative regulatory factors, which can then be validated in vivo.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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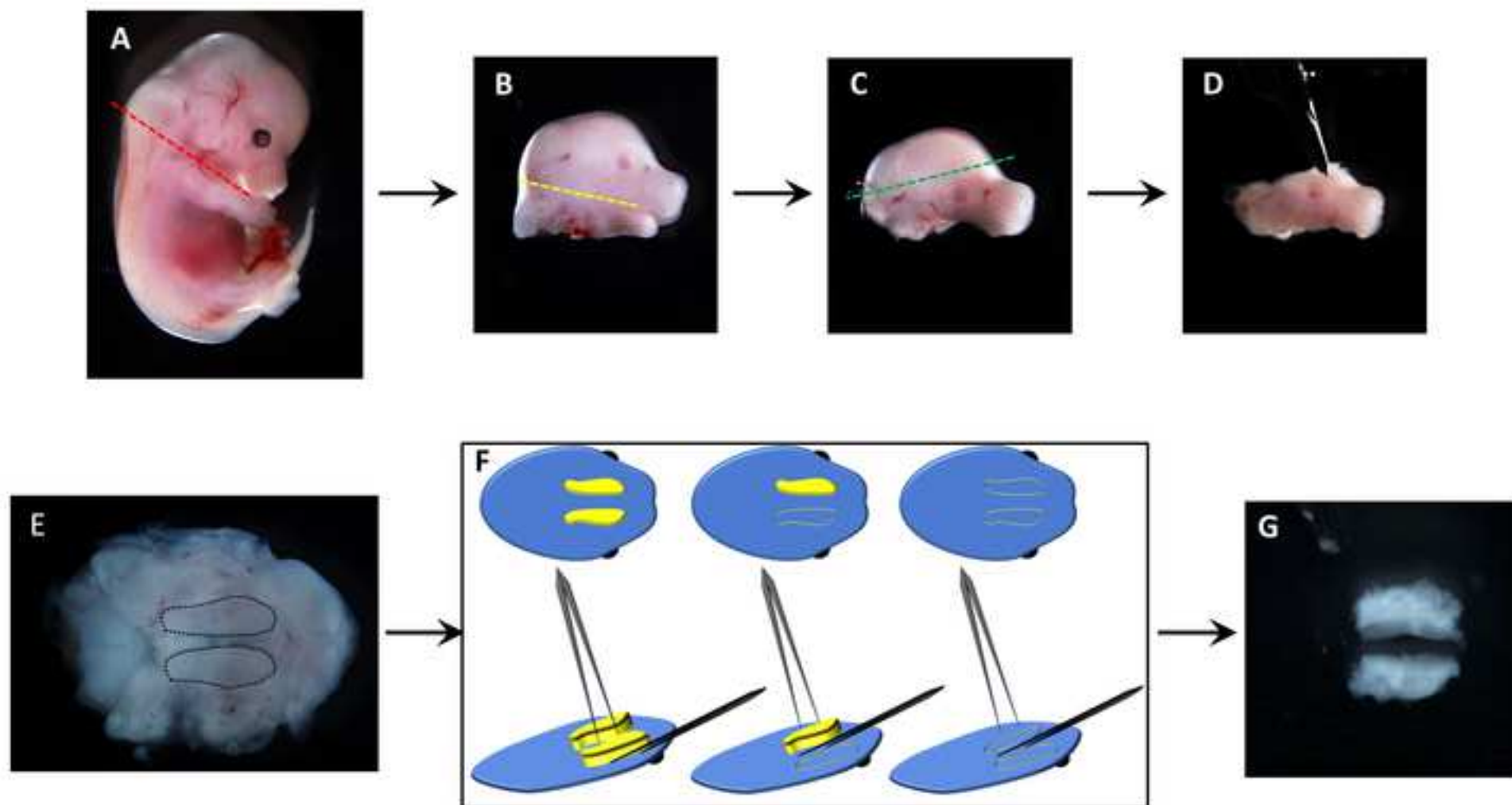
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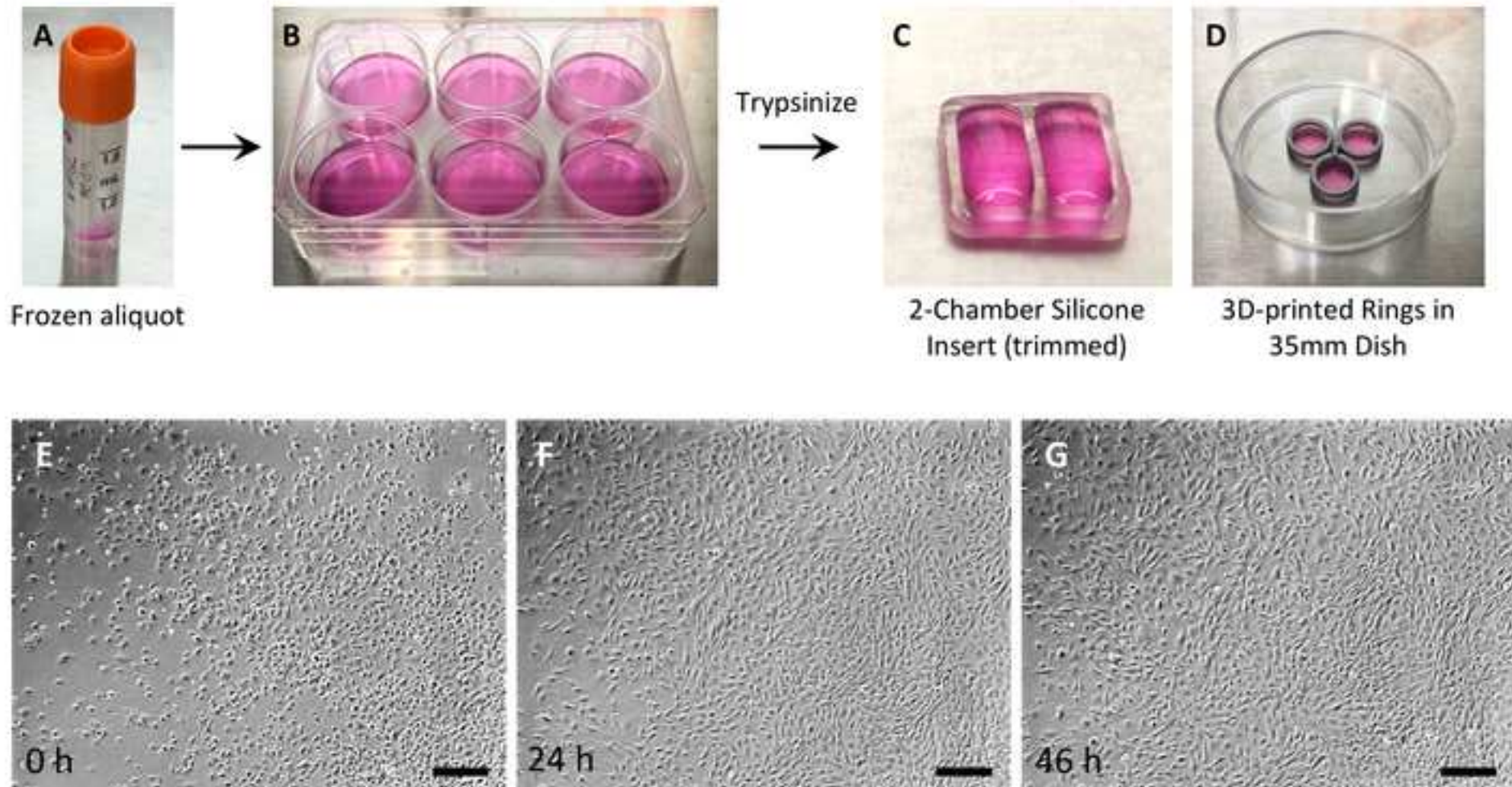
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2D Collective Migration Assay



Wound-Closure Assay

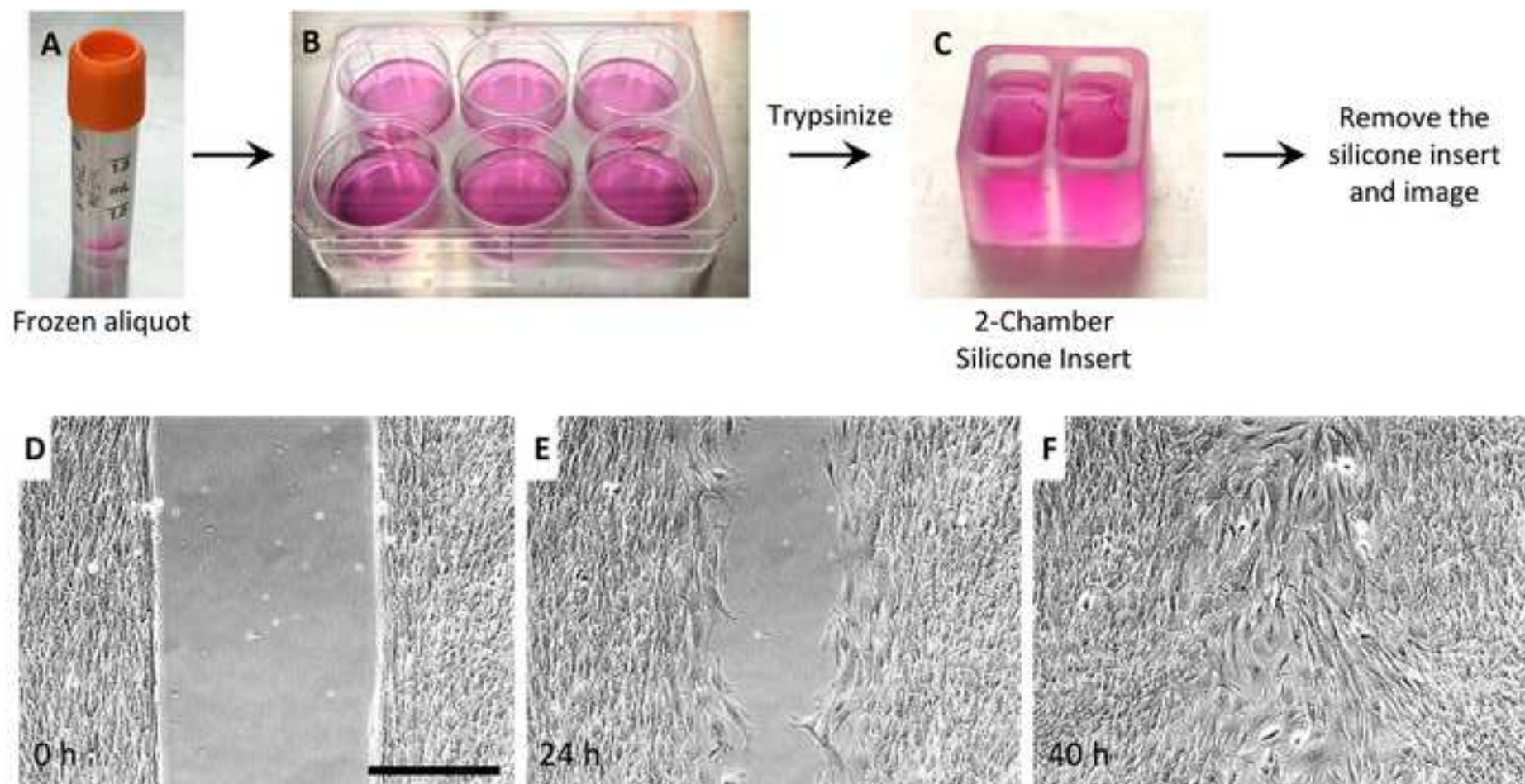


Figure 4

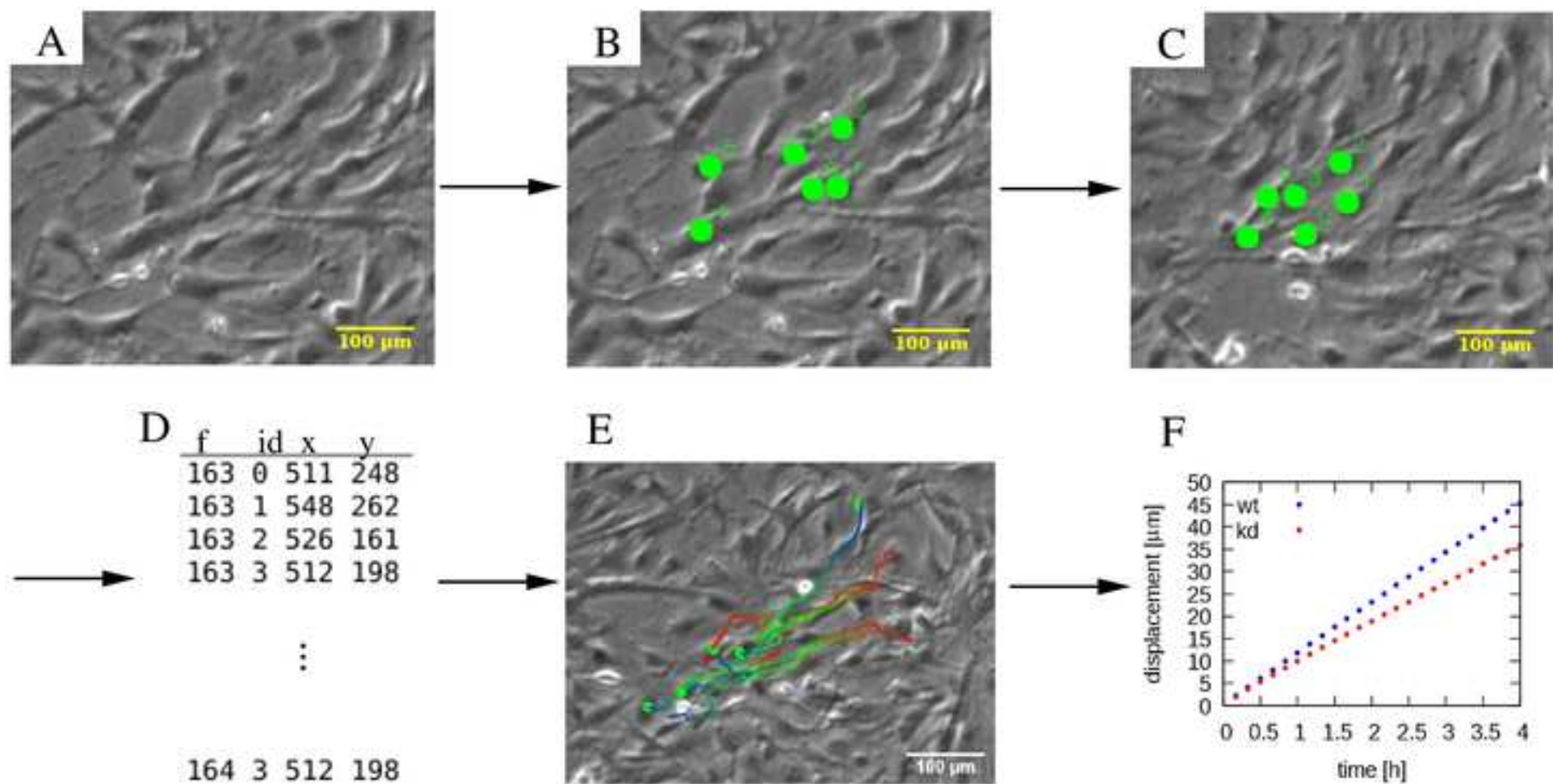
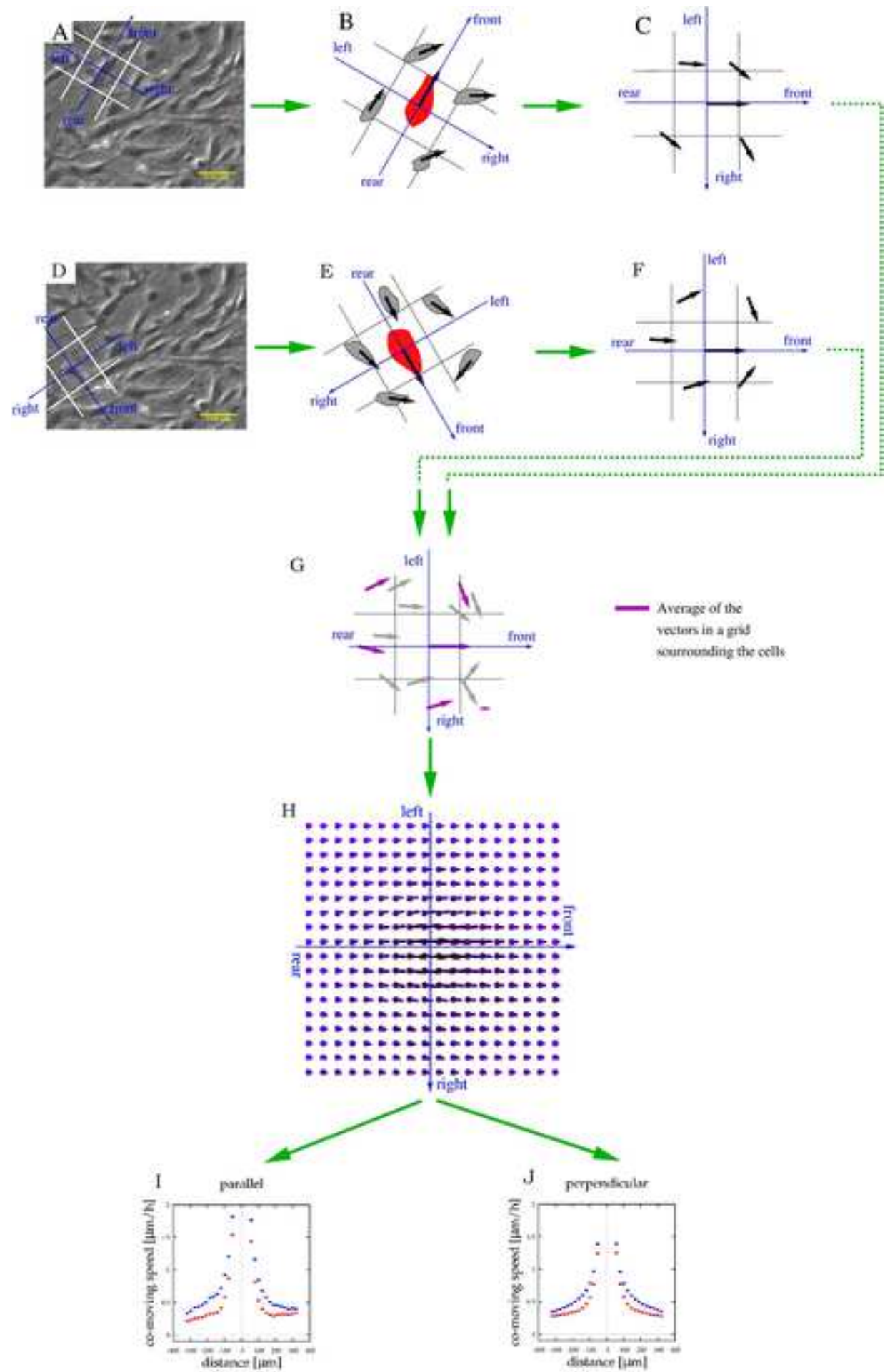


Figure 5



| Name of Material/Equipment | Company | Catalog Number | Comments |
|--|--------------------------|----------------|---|
| Beaker, 250 mL (x2) | Fisher Scientific | FB-100-250 | |
| CO ₂ | Matheson Gas | UN1013 | |
| Conical tubes, 15 mL (x1) | Midwest Scientific | C15B | |
| Debian operating system | | | computational analysis of time-lapse images |
| Dulbecco's Modified Eagles Medium/High Glucose with 4 mM L-Glutamine and Sodium Pyruvate | Cytiva Life Sciences | SH30243.01 | |
| EtOH, 100% | Decon Laboratories | 2701 | |
| EVOS FL Auto | ThermoFisher Scientific | AMAFD1000 | |
| EVOS Onstage Incubator | ThermoFisher Scientific | AMC1000 | |
| EVOS Onstage Vessel Holder, Multi-Well Plates | ThermoFisher Scientific | AMEPVH028 | |
| Fetal Bovine Serum | Corning | 35-010-CV | |
| Fine point #5 Stainless Steel Forceps (x2) | Fine Science Tools | 11295-10 | Dissection |
| Instrument sterilizer bead bath | Fine Science Tools | 18000-45 | |
| Microcentrifuge tubes, 1.5mL | Avant | 2925 | |
| Micro-Dissecting Stainless Steel Scissors, Straight | Roboz | RS-5910 | Dissection |
| NucBlue (Hoechst) Live Ready Probes | ThermoFisher Scientific | R37605 | |
| Penicillin Streptomycin Solution, 100x | Corning | 30-002-CI | |
| Silicone Insert, 2-well | Ibidi | 80209 | |
| Small Perforated Stainless Steel Spoon | Fine Science Tools | MC17C | Dissection |
| Spring Scissors, 4 mm | Fine Science Tools | 15018-10 | |
| Sterile 10 cm dishe(s) | Corning | 430293 | |
| Sterile 12-well plate(s) | PR1MA | 667512 | |
| Sterile 6-well plate(s) | Thermo Fisher Scientific | 140675 | |
| Sterile PBS | Corning | 21-031-CV | |

| | | |
|---------------------------------------|-------------------------|----------|
| Sterile plastic bulb transfer pipette | ThermoFisher Scientific | 202-1S |
| Trypsin, 0.25% | ThermoFisher Scientific | 25200056 |

We thank the reviewers for their valuable comments. We have done our best to address each of their concerns, which has improved the revised manuscript.

Reviewer #1:

Manuscript Summary:

Goering et al. provide protocol for Isolation and Time-Lapse Imaging of Primary Mouse Embryonic Palatal Mesenchyme Cells to Analyze Collective Movement Attributes. The protocol consists of cell isolation part, cell culture and computation workflow to analyze migratory properties of cells.

Major Concerns:

1. The protocol is based on publication, which has not been peer reviewed, yet. The critical publication Goering et al., 2019, is deposited only at bioarchives.

Goering et al. has now been accepted for publication in Scientific Reports (Jan. 04, 2021).

2. There is general lack of characterization and reproducible standardization of primary cells. It is necessary to involve such steps into protocol in order to ensure that isolated palatal mesenchymal cells retain the molecular characteristics of native cells during palate development and not more like MEFs.

We have now included additional morphological, growth and motility characteristics to ensure that the isolated MEPM cells are healthy and undifferentiated. We have now added a new step 3.9.1, a new note following 3.11, and the following information in the discussion:

"It is important to first optimize conditions for culture and assays using only wildtype MEPM cells. After the palatal shelves have been successfully trypsinized and the resulting single-cell suspension plated overnight, the vast majority (~90%) of cells will readily attach to the growth-surface of the dish. If the cells do not readily adhere, then there may be something wrong with the culture conditions. Initially, the adhered cells will look fairly homogenous, with a triangular or slightly elongated shape, but as they grow to a high density they begin to become more spindle shaped and elongated (Figure 2). If the cells become dramatically large in size or multinucleated, they should not be used for experiments.

This optimization will also establish basic wildtype parameters for successful growth (time to confluence) and wound repair (time to closure). The cells should proliferate by doubling in number almost daily, and in time-lapse images show motility of ~5-10 μ m/hour."

3. The concept of analysis morphogenic collective migration by wound healing assay is not biologically relevant.

We believe that the wound-repair assay is still one of the simplest and widely used assay to assess directional movement - an important attribute of collective motility. We agree with the reviewer that additional analyses would help validate biological relevance.

Reviewer #2:

Manuscript Summary:

This manuscript outlines the steps to establish cultures of primary mouse embryonic palatal mesenchyme (MEPM) cells and subsequently live image and quantify 2D cell migration and wound repair assays using these cells. The premise that the method presented can be used to model morphogenic movements of palatal mesenchyme cells contributing to palatal shelf elevation is not supported as written. The protocol is well written, though additional experimental details would strengthen the manuscript.

Major Concerns:

1. The authors should go into more detail in the Introduction on the known roles of the secondary palatal shelf epithelia versus mesenchyme in contributing to palatal shelf elevation. As written, it is not clear that the mesenchyme plays a demonstrated role in this process. Relatedly, the main reference forming the scientific premise that palatal mesenchyme cells display collective movement that contributes to palatal shelf elevation has not been peer-reviewed. Therefore, related statements should be tempered.

We have now added more detail regarding the role of epithelium and mesenchyme during palatal shelf elevation in the introduction.

Furthermore, Goering et al. has now been accepted for publication in Scientific Reports.

2. Step 2.7 is too vague and should be expanded with a description of structures surrounding the secondary palatal shelves. In addition, the contrast in Figure 1E should be improved to better visualize the palatal shelves.

We have added more detail to 2.7 and improved the contrast of Figure 1E. We noticed that the JoVE compiled pdf has lower resolution figures than were uploaded. Please download the larger version of the figure to see full resolution.

3. At the end of Step 3.11 that authors write "NOTE: MEPMs may be passaged up to 3 times" (line 190), but later note that "... we strictly limited the number of MEPM cell passages to two for our experiments" (line 379) and that "Restricting analysis to only 2 cell passages greatly maintains the quality of MEPM cells" (line 402). Therefore, the authors should encourage readers to passage MEPMs up to 2 times or provide additional justification for increased passages.

We agree with the reviewer, and have removed the note - we have consistently seen good results with 2 passages.

4. The authors should provide full names and source information for the software and computational tools used (i.e. with the EVOS FL Auto microscope, python interpreter, C compiler and Debian operating system).

All commercial tools are now listed in the Table, as requested by the Editor. The public tools are now further clarified in the protocol as well as in the Table.

Minor Concerns:

1. Primary MEPM cells have also been used in several instances in migration, proliferation and biochemical studies, in addition to gene expression studies. See Bush and Soriano 2010; He and Soriano, 2013; Fantauzzo and Soriano, 2014; and Mo et al., 2020.

We have now included all of these references.

2. Previous reports of primary MEPM cultures have included pen-strep and L-glutamine in the culture media. The authors should at least touch on why these components are not included here.

We apologize for this oversight - we also use pen-strep and L-glutamine in our MEPM culture medium. We have now clarified this in the protocol.

3. In Step 6.6, after removing the silicone inserts, does additional media need to be added to the well? Presumably there is only 200 uL of media in the well at this point.

Yes, that is correct - we apologize for the omission. We have now clarified to "add 3mL of media to the well before removing the silicone insert".

4. In Step 7.1.1. the authors should define the segmentation threshold as well as what the output is being checked for (line 289).

Done. A threshold needs to be selected that minimizes background error and most closely determines the cell-covered area.

5. In Step 7.1.3. the authors should define "d" in the equation.

Done. d represents delta or difference.

6. Similarly, in Step 7.5 the authors should define "a" in the equation.

Done. a represents one of the parameters of the exponential function fit of the flow field vectors.

7. Lines 366 and 367 refer to Figure 1G instead of Figure 1E.

We have corrected the error.

8. The authors should indicate in the Figure 4 legend how much time has elapsed between B and C or if these are images from independent experiments. Also, the authors should define "kd" and "kd + y".

We have included the information. All panels are from the same experiment.

Reviewer #3:

Manuscript Summary:

This article details the important, step-by-step approach to generating palate mesenchymal cells for culture and experimentation. The MEPM are a great source for evaluating cellular behavior of the palate and has provided many researchers with keen insights into the understanding of cleft palate formation. Using these MEPM, the authors also demonstrate how the cells can be compared in their proliferative and migratory behavior. The authors have developed unique tracking software to coincide with their in vivo tracking and wound closure models.

Major Concerns:

1. in the introduction and the discussion the authors mention that their studies indicated up regulation of PI3k-AKT pathway of Specc1 mutations. this sentence is put in without much context and I would suggest providing some background of other authors that have studied palate cell behavior and how these assays, and the Specc1 study have leveraged this and other modalities of measuring cell migration and spreading.

The use of PI3K-AKT activator is very specific to our Specc1 mutant biology. We only mention it as an example of using various biochemical manipulations in combination with the assays described here. In response to your Concern#6, and Rev.2 minor concern #1, we have now added more examples and references of MEPM use, and included PI3K-AKT activator as another example. Further detail about our Specc1 mutant biology and PI3K-AKT activator effect in these assays can be found in Goering et al., which has now been accepted for publication in Scientific Reports.

2. The authors should also address other reports that indicate palate elevation is a function of hyaluronic acid expression and how these studies reflect or address these hypotheses.

We have now included the hyaluronic acid related references under the category of "extracellular matrix components" that have been shown to affect mesenchymal remodeling.

3. In Figure 1, the authors should demonstrate either with their forceps or schematically, how the palate shelves are removed. The dissection of the palate shelves, leaving the other surrounding tissues is a critical part of the procedure that is technically the most challenging to conceptualize.

We strongly agree with the reviewer that this is a critical part of the procedure, which is why we have included this part in the video component of the paper (highlighted in yellow) as watching it would be the best way to conceptualize and reproduce the procedure. However, we have now also included a schematic (new Fig.1F) demonstrating the removal of the palatal shelves.

4. In figure 2 and 3 the authors should discuss the other alternative ways to perform migration and wound closure assays and describe the advantages of the ibidi 2 chamber model.

We have added this information in the discussion. Briefly, a silicone chamber system allows standardization of the wound width, as well as prevents the formation of a cell ridge due to manual wound creation. Furthermore, for WT and mutant comparison, we prefer to analyze MEPM from individual embryos independently. This results in a very small number of cells, which can only be assessed in a small area that has to be amenable to imaging. The modified silicone

inserts also satisfy that requirement. However, for 2D culture analysis for example, small 3D printed rings can also be used (Figure 2C').

5. In figures 4 and 5 the authors should detail if these assays have been used by other authors and how they have been accomplished here differently with the proprietary software.

We would like to emphasize that these software tools are not proprietary, but open-source, freely available from github from the links provided in the manuscript. We provided references in section 7.

6. furthermore, if the authors could provide suggestions or recommendations on how in situ stimuli (e.g. growth factors) implanted on beads or other delivery vehicles have been used or could be used to determine the cellular behavior of MEPM in response to environmental cues

We have added additional information to the discussion. Briefly, MEPM cells have been used with PDGFRa and EphB1, and we have used them with PI3K-AKT activators (Goering 2019 bioRxiv; accepted to Sci Rep) and with an antiepileptic drug topiramate (Rafi 2020 bioRxiv; revision submitted). We have added these references. To our knowledge, MEPM cells have not been used with beads, however, we do not see why not.

overall this is a good paper that captures the essence of MEPM isolation and will be a benefit to the community.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Done.
2. Please revise the following lines to avoid overlap with previously published work: 351 (Briefly,...)-357; 455 (After repeating...)-460
Done.
3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Done.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Eppendorf; EVOS FL Auto Time-lapse wizard; ThermoFisher; NucBlue etc
Done.
5. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:
 - a. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.
 - b. Please specify the euthanasia method without highlighting any of these steps.

Done.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have now included more details to the protocol steps. However, a detailed click-by-click description of the microscope time-lapse setup requires the naming of the specific commercial microscope and software being used. The protocol currently includes all relevant information as specifically as possible without referring to commercial program-specific actions.

8. Please make sure you include all instruments used for dissection in the Table of Materials and cite this in appropriately places.

Done.

9. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Done.

10. Please discuss all figures in the Representative Results. However, for figures showing the experimental setup, please reference them in the Protocol. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. Hence, please consider moving the last two paragraphs from the representative results to the discussion section. This will address critical points of the protocol and modification/troubleshooting of the technique.

Done.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Done.

12. As we are a methods journal, please ensure that the Discussion covers the following in detail in 3-6 paragraphs with citations:

- a. Critical steps within the protocol
- b. Any modifications and troubleshooting of the technique
- c. Any limitations of the technique

- d. The significance with respect to existing methods
 - e. Any future applications of the technique
13. Please do not abbreviate journal names in the reference list.
- Done.*
14. Please sort the Materials Table alphabetically by the name of the material.
- Done.*