Video Article

Quantitative Whole-mount Immunofluorescence Analysis of Cardiac Progenitor Populations in Mouse Embryos

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Abstract

The use of ever-advancing imaging techniques has contributed broadly to our increased understanding of embryonic development. Pre-implantation development and organogenesis are two areas of research that have benefitted greatly from these advances, due to the high quality of data that can be obtained directly from imaging pre-implantation embryos or ex vivo organs. While pre-implantation embryos have yielded data with especially high spatial resolution, later stages have been less amenable to three-dimensional reconstruction. Obtaining high-quality 3D or volumetric data for known embryonic structures in combination with fate mapping or genetic lineage tracing will allow for a more comprehensive analysis of the morphogenetic events taking place during embryogenesis.

This protocol describes a whole-mount immunofluorescence approach that allows for the labeling, visualization, and quantification of progenitor cell populations within the developing cardiac crescent, a key structure formed during heart development. The approach is designed in such a way that both cell- and tissue-level information can be obtained. Using confocal microscopy and image processing, this protocol allows for three-dimensional spatial reconstruction of the cardiac crescent, thereby providing the ability to analyze the localization and organization of specific progenitor populations during this critical phase of heart development. Importantly, the use of reference antibodies allows for successive masking of the cardiac crescent and subsequent quantitative measurements of areas within the crescent. This protocol will not only enable a detailed examination of early heart development, but with adaptations should be applicable to most organ systems in the gastrula to early somite stage mouse embryo.

Video Link

The video component of this article can be found at https://www.jove.com/video/56446/

Introduction

The study of organogenesis has long relied on the observation of morphogenetic events in the developing embryo. These studies frequently rely on the use of fluorescent dyes or lineage tracing reporters in combination with labeling of defined reference populations.[1] By comparing relative positions of these labels, information can be gleaned on the origin, movement, or ultimate contribution of a population of interest. Transplantation and fate mapping experiments use either morphological landmarks or injection of dyes into non-motile lineages to define the starting point of the cells of interest, which are then examined for contribution to the developed embryo.[2,3,4,5] Genetic lineage-tracing experiments use the same concept with well-defined reporter alleles that are used to label cell populations without experimental manipulation. Key to these approaches is the ability to determine, with high spatial resolution, the locations of the experimental and reference labels. These approaches have yielded outstanding progress in pre-implantation development and explant organogenesis studies.[6,7,8,9]

The developmental events that underlie heart morphogenesis have been increasingly well described in recent years.[10] One of the major discoveries in this area of research is the description of a number of progenitor populations that can be distinguished by expression of unique markers.[11] These populations include the First and Second Heart Fields (FHF and SHF), which are present within the cardiac crescent at the anterior side of the embryo at embryonic day (E) 8.25 of mouse development.[12] These populations are frequently examined through a combination of wide-field microscopy, which provides tissue-level information, and serial sectioning with immunofluorescence assays, which offers high cellular resolution but only two-dimensional spatial information.[13] Thus, while these studies have greatly advanced our understanding

of heart development, the available methods have limited in depth quantitative analysis of morphogenesis during these stages, creating the need for approaches to examine the organization of these populations on a whole-organism level.

The recent advances in both confocal microscopy and 3D image analysis allow for high-resolution and high-throughput algorithmic reconstructions of cells and structures *in situ* with relative ease, thus paving the way for detailed studies of complex cellular structures.[14] With the increase of computational power and the development of big-data managing algorithms, both necessary to handle the exponential increase of the size of imaging data-sets, analyses can now be fully automated.[15] Automated analysis of imaging data-sets has the benefit of being unbiased, but it is only as reliable as the quality of the input dataset; it is imperative, then, that best-practices are used during acquisition and image pre-processing to ensure the highest quality, unbiased analysis.[16] Protocols can be completely automated and shared for reproducibility, and the algorithms used by proprietary software are readily available through libraries to be used by scientists who have familiarity with modern proprietary or open-source developer tools.[17]

The following protocol explains the necessary steps to perform such analysis on one well defined model of organogenesis, the formation of the cardiac crescent during heart development. Specifically, this protocol describes how to (1) harvest and dissect cardiac crescent stage embryos, (2) perform whole mount immunofluorescence for reference (Nkx2-5) and experimental (Foxa2Cre:YFP[18,19]) markers, (3) prepare and image the embryos using confocal microscopy, and finally (4) analyze and quantify the resulting images using advanced three-dimensional approaches. While the cardiac crescent is used as an example here, with appropriate modification, this protocol may be used for analysis of multiple lineages in gastrula to early somite stage embryos.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

1. Harvesting and Processing Cardiac Crescent Stage Embryos

1. Mate a fertile female mouse with a fertile stud male.
2. Check for the presence of a vaginal copulation plug using a blunt probe or forceps. Noon on the day of plug detection is considered embryonic day (E) 0.5 (See **Figure 1A** for full timeline).
   NOTE: Plugs should be checked for in the morning, as they are lost throughout the day.
3. On the morning of the 8th day (E8.25), sacrifice the pregnant dam by CO_2 inhalation, or according to local and institutional regulations.
   NOTE: Exact timing can be strain dependent and should be determined empirically by morphology.
4. Spray the abdomen of the mouse with 70% ethanol to clean the area and minimize shedding. Expose the viscera by performing an abdominal incision through both the skin and body wall.
5. Locate the uterus and carefully remove the entire uterine horn from the animal. Start by cutting above one oviduct, trimming fat away from the uterus while proceeding to the cervical end. Cut through the cervix and continue to the upper end of the other oviduct, cutting to release the entire uterus.
6. Place the uterus in a 10 cm dish with phosphate buffered saline (PBS, pH 7.4) to wash away excess blood. Sub-dissect the uterus by cutting the mesometrium between each deciduum, which contains the embryos. Transfer the embryos to a 6 cm dish with fresh PBS.
7. Under a dissection microscope, use fine forceps (#5) to remove the uterine tissue away from the decidual tissue.
8. Using forceps, carefully slice the tip of the embryonic half of the deciduum to reveal the embryo. Pinch the deciduum to push the embryo out and carefully pull the embryo out.
9. Dissect away extraembryonic tissues as much as possible without damaging the morphology of the embryo (**Figure 1B**).
10. Use a transfer pipette to place the embryos in a 1.5 mL tube with fresh PBS and place on ice. Repeat 1.7-1.9 for the remaining embryos before continuing.
11. Aspirate PBS and rinse once with PBS. Aspirate PBS.
    NOTE: Try to remove as much PBS as possible without damaging or drying the embryos. Manual removal of solutions is recommended at all steps to avoid loss of embryos.
12. Fix embryos with 4% paraformaldehyde (PFA) in PBS for 1 h at RT.
    NOTE: Embryos can be fixed overnight (O/N) at 4 °C.
13. Rinse three times with PBS. Keep embryos at 4 °C until ready to proceed with immunofluorescence.
    NOTE: Pause point. Embryos can be safely stored in PBS at 4 °C for several weeks.

2. Immunofluorescence Staining

NOTE: The incubation conditions below can be adjusted to accommodate different schedules. Use of gentle shaking or rocking for all long incubation steps is recommended.

1. Remove PBS and add 1 mL of blocking buffer (0.5% saponin, 1% bovine serum albumin (BSA) in PBS).
2. Incubate at least 4 h at RT. This can also be done O/N at 4 °C.
3. Remove blocking buffer and add primary antibody mixture diluted in blocking buffer. Incubate O/N at 4 °C.
   NOTE: See Materials section for description of antibodies used and suggested dilutions. Antibody dilutions should be determined empirically. The use of Nkx2-5 as a reference stain for the cardiac crescent is recommended, and is key to downstream image segmentation and analysis steps.
4. Remove primary antibodies by aspiration.
5. Wash 3 times for 1 h each with 0.1% Triton in PBS.
6. Remove wash and add secondary antibody mixture diluted in Blocking buffer. Incubate for 3 h at RT. This can also be done O/N at 4 °C.
7. Wash 3 times for 1 h each with 0.1% Triton in PBS. This can also be done O/N at 4 °C.

8. Counterstain with 4',6-diamidino-2-phenylindole (DAPI) in PBS for 10 min.
   NOTE: This counterstain can be performed simultaneously with secondary antibody.
9. Wash 2 times for 5 min each with 0.1% Triton in PBS.
10. Slowly suspend embryos in anti-fade mounting media (2% w/v n-Propyl gallate (nPG), 90% glycerol, 1x PBS; See Materials section for more details). Allow to equilibrate at least 1 h before mounting. Gently flick tube periodically to help embryos drop into anti-fade solution.
    NOTE: Pause point. Embryos can be stored for several days in anti-fade solution until ready to mount and image.

3. Mounting Embryos for Microscopy

1. Prepare microscope slides for mounting using either double-stick tape or silicone spacers. If using double-stick tape, make two parallel stacks of 5-6 layers about 15-20 mm apart. This will leave enough space to place the embryos and secure the coverslip (**Figure 1C**).
2. Place a 15 µL drop of anti-fade on the slide, between the double-stick tape. Carefully transfer one embryo to the slide.
   NOTE: More than one embryo can be placed on a slide. However, the subsequent orientation steps are more difficult with multiple embryos, and long imaging durations may lead to photo-bleaching.
3. Under a dissection microscope using fine forceps, position the embryo such that the anterior side faces away from the slide with the body axis in line with the long axis of the slide. See **Figure 1C** for a schematic of the mounting setup.
4. Carefully place a coverslip over the sample by resting one side on one stack of double-stick tape and using forceps to gently lower the coverslip until it contacts the other tape.
   NOTE: Steps 3.3 and 3.4 are critical to ensure the correct orientation of the embryo for imaging. By lowering the coverslip along the posterior to anterior direction, the embryo should not roll and the cardiac crescent region will stay oriented away from the slide, which is ideal for imaging on an inverted microscope.
5. Use a pipette to add additional anti-fade between the slide and coverslip to keep the sample from dying out during storage/imaging.

4. Confocal Imaging

1. Acquire images using the highest magnification objective that allows for the whole cardiac crescent region to be captured in one field of view. Use Nyquist sampling rates to determine x-y-z voxel dimensions. Most microscope manufacturers will have an "optimize" button to ensure Nyquist sampling.
   NOTE: Staining for Nkx2-5 is beneficial here, as it will delineate the entire cardiac crescent region. Smaller Z-step sizes (oversampling) will yield better 3D modeling results but may lead to extended acquisition times and/or bleaching for some samples.
2. Set up imaging parameters using standard best-imaging practices. Namely, use the lowest possible laser intensity to achieve good signal-to-noise ratio for each fluorophore and choose gain and offset levels that yield a wide dynamic range without saturating the signal.
   NOTE: Be consistent with imaging parameters between samples that will be directly compared. This is especially important for downstream 3D analysis and quantification.

5. Image Analysis and Quantitative 3D Modeling

NOTE: For this protocol, images were analyzed using the Imaris software package. Similar analysis may be possible using alternative packages. The description below covers the analysis pipeline for a reference channel (*i.e.* Nkx2-5) and one experimental channel (*i.e.* YFP). Additional channels can be analyzed through repetition of these steps. An example dataset has been provided that can be used to replicate the analysis below.

1. Load raw image data sets into Imaris Arena view and open file for desired embryo in Surpass view. Data should load in 3D View in MIP (max) mode. Open the Display Adjustment window and select only the reference channel.
2. Adjust the threshold of the image if needed for better visualization. For this analysis, adjust the gamma if the signal-to-noise ratio is too low for segmentation.
   NOTE: If performing gamma corrections, which are non-linear adjustments, the adjusted images cannot be used for intensity measurements or comparisons. At this stage you can perform further image pre-processing (i.e. illumination corrections, Gaussian blur or other filtering), if your signal-to-noise ratio is not satisfactory. If you choose to do pre-processing, you must perform the same pre-processing steps for all images in your dataset.
3. **Create a new surface for the reference channel using the Surface algorithm dialogue.**
   1. Select the "Segment only a Region of Interest" checkbox.
   2. Adjust the bounding box region so that it contains the region of interest (ROI, *i.e.* the cardiac crescent area) as delineated by the reference stain.
   3. Select the reference channel from the Source Channel dropdown menu. The default setting for Smoothing (equal to the z voxel size) is typically sufficient but may require empirical optimization. For this analysis, use smoothing equal to half the z voxel-size.
   4. Perform thresholding by absolute intensity. To ensure that the surface does not extend beyond the channel signal, adjust the lower limit using the sliders.
      NOTE: For certain images where the cells can be delineated easily, you can include the "split touching objects" algorithm, to further separate your signal from background.
   5. Filter objects by voxel number or volume, and reject background (small or found in areas outside of the Nkx2-5 area) objects that the algorithm might have generated. Finish the algorithm.
4. **Using the newly created surface, create a masked channel for the experimental channel.**
   1. With the surface(s) selected, choose the "Mask Sel ..." function from the Edit window.
   2. Select the experimental channel of interest (*i.e.* YFP). Make sure that the "Duplicate channel before applying mask" checkbox is selected.

5. In the Display Adjustment window select only the masked channel. Create a new surface for the masked channel by following steps 5.3-5.3.5.
   NOTE: At this point it may be helpful to adjust the appearance of each mask to facilitate the visualization of the 3D model: Select each surface and select the color tab. Adjust the Base color and transparency as needed. Surface colors do not merge when overlapped.
6. To obtain the volumetric data, with each surface selected select the Statistics tab. In the "Detailed" sub-tab, choose "Average Values" from the drop-down menu. The total volume of the surface can be found in the Sum column of the generated table.
   NOTE: If the generated surface contains erroneous fragments (*i.e.* fragments that are from background signal not excluded during segmentation or areas discontinuous from the main surface), it may be necessary to calculate the total volume manually, or filter the surfaces by volume or number of voxels and ensure that only signal segments are included in the calculation. The volume for each portion of the surface can be found by selecting "All Values" from the drop-down menu. Selecting each value will make the corresponding region appear yellow, which aids in determining values to exclude.

Representative Results

The quality of the final data and analysis depends greatly on (1) the integrity and morphology of the dissected embryos, (2) the use of high specificity antibodies, and (3) the proper setup of imaging parameters. Damaged embryos will confound the surface generation process and hinder quantitative analysis. Examples of properly dissected and staged embryos are shown in **Figure 2B**. The embryos can be further dissected by removing the yolk sac, to which antibodies will frequently bind non-specifically. However, removal of the yolk sac will make the embryos less robust, so care should be taken during downstream handling.

The combination of high quality antibodies and proper imaging settings are crucial to achieve high signal-to-noise ratio images. **Figure 2A** shows an example image using antibodies against Nkx2-5 as a reference marker to label the cardiac crescent, and GFP to label a lineage traced population of interest (Foxa2Cre:YFP ventricular cardiovascular progenitor cells) (See supplemental data for raw image and data files).[19] This strategy allows for the comparison of a given population with the entire cardiac crescent region. For some experiments, it may be of interest to examine the FHF and SHF subdomains of the cardiac crescent. In this case, the FHF and SHF can be labeled with Hcn4 and Islet1, respectively (see reference[19] for images and quantitative analysis with these antibody combinations). With the appropriate setup and secondary antibody combinations, we have successfully imaged and analyzed three lineages concurrently (*i.e.* the cardiac crescent, YFP, and first or second HF).

Inability to achieve strong signal will also limit the ability to generate high confidence 3D models, as it will become difficult to remove background signal from the final surfaces. In these cases, care should be taken when adjusting image gamma (during pre-processing, **Figure 2B**, **2C**, **2N**, **2O**) or surface thresholding (during surface generation algorithm, **Figure 2G**, **2H**, **2L**) and similar settings should be used for all images within an experiment. Failure to do so will result in inconsistent volumetric data that would be inappropriate to use for direct comparison. Often, erroneous surfaces resulting from background signal can be removed through size filtering (**Figure 2I**, **2J**), though doing so from high-background images will be difficult without losing true surface fragments as well.

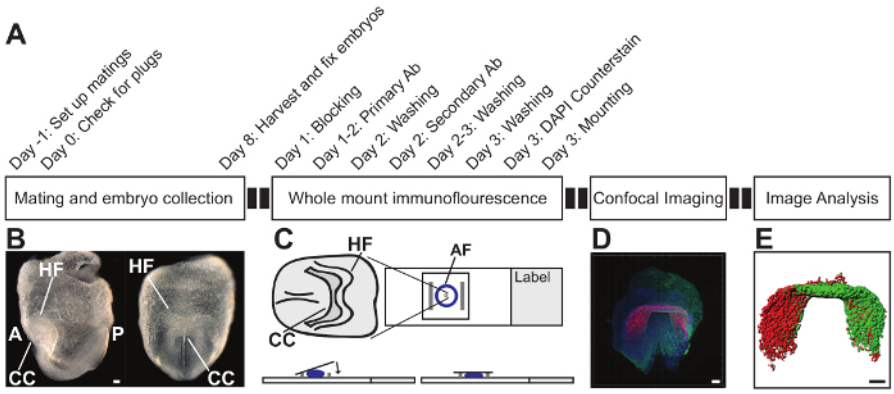

Figure 1: Experimental timeline and schematics. (**A**) The entire experiment, from mating to data analysis, can be carried out in approximately two weeks. Embryos are collected in the morning on the 8th day post copulation (E8.25) for cardiac crescent stage analysis (**B**). Once fixed, embryos are blocked and stained with primary and secondary antibodies before mounting. Embryos are mounted with standard microscopy slides and coverslips, using double-sided tape as a spacer (**C**). Embryos are placed in a drop of anti-fade in the displayed orientation (C, upper) and a coverslip is slowly lowered onto the tape (C, lower). Confocal imaging and image analysis are performed to generate high resolution z-stack images (D) and 3D surface models (**E**). HF, head fold; CC, cardiac crescent; A, anterior; P, posterior; AF, anti-fade. Scale bars are 100 µm.
Please click here to view a larger version of this figure.

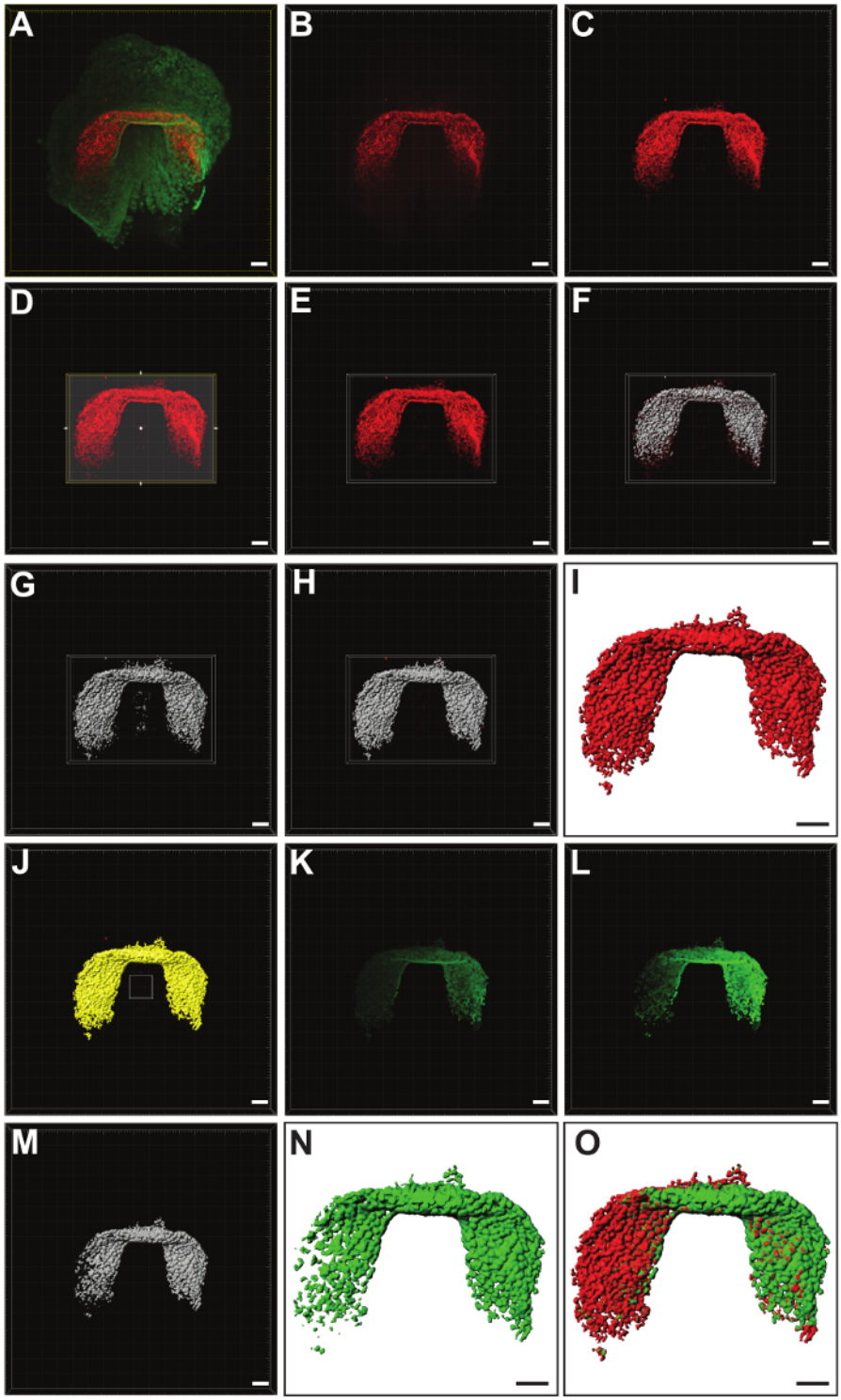

Figure 2: Stepwise confocal image processing and 3D surface generation. (**A**) Confocal z-series loaded in the volume view. The reference channel (Nkx2-5) is selected (**B**) and the intensity and gamma adjusted (**C**) before starting the Create Surfaces algorithm. After the region of interest is selected (**D**), and the level of surface detail is chosen (**E**). The initial surface (**F**) is thresholded to include all true signal in the surface (**G**). Filtering is then performed to remove small background fragments (**H**) to yield a final reference surface (**I**). By selecting the reference surface (**J**), the comparison channel (YFP) can be duplicated and masked (**K**). The intensity and gamma for this channel are then adjusted (**L**) before generating a second surface through the same sequence of steps (**M, N**). The total volume for each surface is calculated automatically and can be compared both quantitatively and visually (O, note that surface colors do not merge when overlapped). Scale bars = 100 µm. Please click here to view a larger version of this figure.

Discussion

The protocol above describes a strategy for obtaining quantitative data from high quality whole mount immunofluorescence images of post-implantation mouse embryos. When performed correctly, the 3D volumetric data generated through these steps can be used for comparative and intersectional analysis of multiple domains within the embryo. The surface signal masking approach described is of particular use when investigating novel cell populations in comparison with well-established reference structures.

We believe that this approach offers a distinct advantage over existing approaches. Whole mount immunofluorescence with wide-field imaging can offer tissue-level information but lacks cellular resolution. Conversely, immunofluorescence of serial sections can give detailed cellular-level resolution, though these data lack the three-dimensional advantage of whole mount imaging. While three-dimensional confocal imaging addresses these shortcomings, few users take advantage of the quantitative potential of this data, and we hope that our protocol will allow more researchers to take full advantage of the data they collect.

Here, this approach has been exemplified using the marker Nkx2-5 to delineate the cardiac crescent and examine the presence of Foxa2Cre:YFP lineage-traced cells within that domain. However, due to the ability to image at least three lineages concurrently and the high resolution three-dimension data generated, this protocol will likely be useful to researchers in many fields. We propose that this approach can be applied to multiple embryonic stages, organ systems, and lineages through minor adjustments to the protocol based on the specific needs of the system. In our experience, this protocol is directly applicable to a range of embryonic ages (E6.5-E9.5 at minimum) with limited adaptations.[19] We have not encountered issues with permeability or antibody penetration in embryos as large as E9.5 with the incubation times listed, though we expect that with thicker or denser tissues these would have to be elongated to achieve full penetration.

The analysis described here was performed using a proprietary software package, but can be adapted to be performed in alternative proprietary packages optimized for big-data handling. Alternatively, similar pipelines can easily be implemented using computational languages or open-source software packages, which will lead to the rapid development of a shareable protocols database. The growth of a global network of scientists, with access to freely available tools and protocols for image acquisition and analysis, will rapidly advance the field and increase the reproducibility of studies.[17]

In our experience, the major limitation to this approach was related to imaging depth, especially for later embryonic stages, and will depend heavily on the specifics of the microscopy setup being used. Similarly, how samples are mounted for imaging will depend on the specific needs of the user. We have found that placing the region of interest closest to the lens improves imaging depth and decreases background signal, and we suggest using layered double-sided tape for mounting, as it enables users to create a chamber that fits their specific needs. Use of a glycerol-based mounting media with an anti-fade reagent, and equilibrating samples in this media before mounting, is highly recommended to suppress photobleaching. Finally, the samples used in this analysis are nearly transparent; users may need to incorporate clearing steps for more advanced embryonic stages, tissue fragments, or organoids, and determining the best clearing protocol for a given application will require empirical testing.

In the case of cardiac morphogenesis, various imaging methods can be used. While confocal microscopy is used here, light-sheet microscopy is extremely well suited for these studies because of the speed, resolution, and signal-to-noise ratio of the acquired image.[20] We expect the increased availability of this new technology to further advance the utility and richness of similar imaging approaches. Similarly, the continuous advancement of fluorescent reporters,[21] chromophores, dyes,[22] and live-embryo culture[23,24] will lead to these studies moving to four dimensions, adding the element of time, and yielding yet more information about how embryonic structures form during development.

Disclosures

The authors have nothing to disclose.

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