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High Resolution Imaging of Mouse Embryos and Neonates with X-Ray MicroCT

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Abstract

Iodine-contrast microCT 3D imaging provides a non-destructive and high-throughput platform for studying mouse embryo and neonate development. Here we provide protocols on preparing mouse embryos and neonates between embryonic day 8.5 (E8.5) to postnatal day 4 (P4) for iodine contrast microCT imaging. With the implementation of STABILITY method to create a polymer-tissue hybrid structure, it has been demonstrated that it not only minimizes soft tissue shrinkage, but also decreases the minimum required soft tissue staining time with iodine, especially for E18.5 to P4 samples. In addition, we also provide a protocol on using commercially available X-CLARITYTM hydrogel solution to create the similar polymer-tissue hybrid structure on delicate early post-implantation stage (E8.5 to E14.5) embryos. With its simple sample staining and mounting processes, this protocol is easy to adopt and implement for most of the commercially available, stand-alone microCT system in order to study mouse development between early post-implantation to early postnatal stages.

Keywords

MicroCT; Iodine-contrasting; STABILITY; X-CLARITYTM

INTRODUCTION

Micro-computed tomography (microCT) has been demonstrated to provide a non-destructive method for studying the morphological and structural changes of mouse embryos and neonates during development (Wong et al., 2014; Dickinson et al., 2016; Hsu et al., 2016; Ermakova et al., 2018). Acquired 3D volumes can be used for morphological analysis, as

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Basic Protocol 1: Generating hydrogel-tissue hybrid of mouse embryos and neonates

Support Protocol 1: Prepare early post-implantation mouse embryos (E8.5 to E14.4) with X-CLARITYTM-hydrogel solution

Basic Protocol 2: Sample staining, sample mounting, data acquisition, and data reconstruction on microCT

Support Protocol 2: Performing the hydrogel crosslinking with common laboratory equipment

well as provides clear histology-like definition for identifying anatomical features through 2D virtual sectioning (Figure 1). In this protocol, we describe the sample preparation methods for preserving mouse embryos and neonates at different stages as well as the parameters for iodine contrast microCT imaging. Basic protocol 1 introduces two sample preparation methods, STABILITY (for E15.5 and above) and X-CLARITY™ (for E14.4 and younger), to prepare samples for iodine contrast microCT imaging. By creating a hydrogel-tissue hybrid structure, sample shrinkage during iodine staining is minimized and the minimum staining time is shortened for achieving sufficient iodine contrast (Wong et al., 2013b; Hsu et al., 2016). Basic Protocol 2 describes the procedures for sample staining, sample mounting, and imaging parameters for different stages of mouse embryos and neonates, as well as data reconstruction for visualization and analysis. The mounting method and imaging parameters introduced in this protocol are based on using a commercially available microCT system Skyscan1272 from Bruker (Figure 2) and the software Nrecon (Bruker) for tomographic image reconstruction. Samples prepared by STABILITY in Basic Protocol 1 have also been imaged via X-Ray Microscope Xradia Versa (Zeiss). Samples processed using the steps from this protocol can be imaged on other microCT instruments by using software package provided by manufacturers. Image processing software, such as CTVox (Bruker), Fiji (NIH), Imaris (Bitplane), Slicer (www.slicer.org), or Vision4D (Arivis) can be used for rendering the reconstructed 3D volume data and data analysis.

BASIC PROTOCOL 1

Generating hydrogel-tissue hybrid of mouse embryos and neonates

MicroCT imaging for studying mouse embryo development has been tested with several different contrast agents, such as osmium tetroxide, phosphotungstic acid, and iodine-based stains (Lugol solution, iodine potassium iodide, 0.1N iodine, etc.) (Metscher, 2009b; Wong et al., 2013b; Hsu et al., 2016). However, the sample staining process depends on penetrance of the contrast agents into the tissue through diffusion to generate contrast on X-ray tomography. This process can take from days to weeks to achieve sufficient penetration throughout the sample and generate contrast homogeneously. In addition, contrasting by iodine will cause different tissue types to shrink at different rates (ex. brain versus muscle); this rate of shrinkage depends on tissue composition, as well as affecting the total volume of the sample. By forming a hydrogel-tissue hybrid structure not only reduces sample shrinkage during iodine contrasting, it can decrease the minimum required staining time for iodine penetration to generate homogeneous contrasting (Wong et al., 2013b; Hsu et al., 2016).

Here we present with two procedures, STABILITY (E15.5 and above) and X-CLARITY™ (E14.5 and younger), to better preserve sample integrity and shorten sample staining time. Although early post-implantation stage embryos can be contrasted with iodine and imaged to resolve the overall morphology and internal anatomic features for phenotyping analysis without being scaffolded with polymer (Hsu et al., 2016; Ermakova et al., 2018), these embryos are prone to damage. Support Protocol 1 adopts a commercially available X-CLARITY™ hydrogel solution to protect the fragile early post-implantation embryos during

the iodine contrasting stage as well as to minimize sample shrinkage in order to preserve the volume information and sample integrity.

Materials—Paraformaldehyde (Sigma, Cat# P6148)

Acrylamide solution, 40% (BIO-RAD, Cat# 1610140)

Bis solution, 2% (BIO-RAD, Cat# 1610142)

VA-044 (Wako Chemical, Cat# 017-19362)

Saponin (Sigma, Cat# 84510)

STABILITY solution (see recipe in Reagents and Solutions)

Scintillation vials, 20 ml (VWR, Cat# FS74512)

Sodium azide (Sigma, S2002)

Fisherbrand™ nutating mixer (Fisher Scientific, Cat# 88-861-041)

X-CLARITY™ hydrogel polymerization system (Logos Biosystem, Cat# C200001)

Sample collection and fixation of mouse embryos and neonates

1. Euthanize timed mated pregnant dam or neonates according to approved protocol from local Institutional Animal Care and Usage Committee (IACUC).
2. Harvest embryos in warm 1X phosphate buffered saline (1X PBS, pH 7.2) to gently remove yolk sac and dissect placenta away from embryo. Early post-implantation stage embryos can also be harvested from the uterus of the pregnant dam within intact decidua or yolk sac and processed for imaging.
3. After dissection, immerse each embryo in individual sample tube with sufficient amount of ice-cold paraformaldehyde (PFA, 4% w/v in 1X PBS, pH 7.2).
4. For neonates, samples can be immersed in ice cold 4% PFA in individual scintillation vial after properly euthanized with locally approved protocol.
5. Perform fixation on a nutating mixer at 4 °C. The recommended volume of fixative and fixation time is based on sample size (Table 1).

Note: The suggested amount of fixative and fixation time is to ensure different stages of mouse embryo and neonates are fixed properly to prevent undesired decomposing of the tissue and organ before imaging.

6. To process mouse embryos (E15.5 and older) and neonates (P0 to P4) with STABILITY hydrogel solution after fixation, prepare fresh, ice-cold STABILITY hydrogel solution for each batch of samples.

Note: *STABILITY* buffer contains 4% w/v PFA, 4% w/v acrylamide, 0.05% w/v bis, 0.25% VA044 initiator, 0.05% w/v Saponin in 1x PBS, pH 7.2. The detail recipe is listed in Reagent and Solution section.

7. Transfer each sample to a 50 ml conical tube with 20 ml of *STABILITY* buffer, immerse samples for 3 days at 4 °C for the polymer to fully penetrate through sample via diffusion.
8. After 3 days of immersion, loosen the cap and place it on top of each conical tube. Perform the crosslinking reaction in a X-CLARITY™ hydrogel polymerization system set at −90 kPa, 37 °C, for 3 hours (Figure 3A and B).

Note: *We use commercially available system to perform the hydrogel polymerization reaction. Alternatively, we also routinely use a desiccator and laboratory built-in benchtop vacuum to remove oxygen, and replaced with nitrogen in the sample tube before incubating at 37 °C within a water bath or incubator for sample crosslinking (Figure 3C)(Hsu et al., 2016). The detail method and common laboratory equipment of this alternative approach is listed as Support Protocol 2.*

9. After the reaction is completed, the *STABILITY* hydrogel solution will solidify, and sample will be embedded within crosslinked *STABILITY* hydrogel (Figure 3D and E).
10. Use a spatula to remove the embedded sample and hydrogel from the tube in a chemical fume hood (Figure 3E and F).
11. Gently remove the crosslinked polymer away from the sample on a dampened paper towel with 1X PBS in a chemical fume hood.
12. Properly discard the crosslinked hydrogel and residual solution in a biosafety container according to local regulation for disposal.
13. Transfer the hydrogel scaffolded sample to a scintillating vial, wash with 20 ml 1X PBS twice, one hour each. Wash with fresh 1X PBS again overnight at 4 °C.

Note: *This step is to make sure any non-crosslinked polymer and VA-044 initiator are removed completely from the sample to prevent further reaction during the iodine staining step.*

14. Remove PBS from the vial and store the stabilized sample with fresh 20 ml 1X PBS with 0.1% sodium azide at 4 °C.

SUPPORT PROTOCOL 1

Prepare early post-implantation mouse embryos (E8.5 to E14.4) with X-CLARITY™ hydrogel solution

This section describes the method of creating the hydrogel-tissue hybrid for samples that are too fragile to be embedded and removed from the *STABILITY* hydrogel after crosslinking. A commercialized tissue clearing procedure X-CLARITY™ from Logos biosystems modified the hydrogel embedding recipe, where the tissue still will undergo immersion in

the hydrogel solution and heat induced crosslinking between the polymer and tissue, but the hydrogel solution will only become viscous instead of solidifying around the sample (Figure 4). This property makes it possible to scaffold the fragile samples with hydrogel polymer to reduce sample shrinkage (Figure 5), preserve sample integrity and reduce the risk of damaging the samples. If desired, the X-CLARITY™ hydrogel solution protocol can also be used for processing E15.5 to E18.5 embryos and neonates as well. Follow the sample collection and fixation (steps 1 to 6) listed in Basic Protocol 1 before starting the procedure listed here in Support Protocol 1.

Materials

X-CLARITY™ hydrogel solution (see recipe in Reagents and Solutions)

Scintillation vials, 20 ml (VWR, Cat# FS74512)

Sodium azide (Sigma, S2002)

Fisherbrand™ nutating mixer (Fisher Scientific, Cat# 88-861-041)

X-CLARITY™ hydrogel polymerization system (Logos Biosystem, Cat# C200001)

Sample scaffolding with X-CLARITY™ hydrogel solution

1. Prepare fresh ice-cold X-CLARITY™ hydrogel solution (see recipe in Reagents and Solutions).
2. Put each sample in individual well of a 12-wells plate with 1 to 3 ml of the X-CLARITY™ hydrogel solution depending on the embryo stages. Incubate at 4 °C overnight.
3. Perform the crosslinking reaction in the hydrogel polymerization system at −90 kPa, 37 °C, for 3 hours (Figure 3A and B).
4. After the crosslinking reaction, the X-CLARITY™ hydrogel solution will become viscous instead of solidifying as gel (Figure 4).
5. In a chemical hood, aspirate and discard the X-CLARITY™ hydrogel solution with a transfer pipet.
6. Properly discard the crosslinked hydrogel in a biosafety container according to local regulation for disposal.
7. Depending on the developmental stage of the embryo, wash the crosslinked sample twice with 1 to 3 ml 1X PBS, 1 hour each, on a nutator at 4 °C. Wash again with fresh 1X PBS overnight at 4 °C.

Note: This step is to make sure any non-crosslinked polymer and initiator are removed completely from the sample to prevent further reaction when perform iodine staining step.

8. Remove PBS from each well and store the polymer-scaffolded sample with fresh 5 ml 1X PBS with 0.1% sodium azide at 4 °C. The crosslinked samples can be store at 4 °C for at least a month before further processing.

BASIC PROTOCOL 2

Sample staining, sample mounting, data acquisition, and data reconstruction on microCT

Here we describe the protocol on using 0.1N iodine solution as contrast agent for microCT. We list the minimum required staining time for each stage of mouse embryo and neonate. We also recommend a simple mounting method by embedding samples in 1% agarose for imaging. The advantages of mounting samples in agarose not only to keep samples hydrated during data acquisition, but also helps to position the samples and reduce random sample movement. We describe the data acquisition process on a Bruker Skyscan 1272 microCT scanner, with Skyscan operation software (version 1.1.13) running on a 64-bit Windows computer as supplied by the manufacturer. We also introduce Nrecon (Bruker, version 1.6.9.8) for tomographic image reconstruction. Samples processed with STABILITY describe in Basic Protocol 1 have also been imaged by X-Ray Microscope Xradia Versa (Zeiss). Samples processed following this protocol can also be imaged on other commercially available microCT instruments with manufacturer supplied software.

Materials

Iodine solution, 0.1N (Sigma, Cat# 38060).

Agarose (Fisher Scientific, Cat# BP160).

Transport tubes with screw caps, 6ml (VWR, Cat# 89497-738)

Skyscan1272 Control software (Bruker, version 1.1.13)

NRecon Reconstruction software (Bruker, version 1.6.9.8)

Sample staining with 0.1 N iodine solution

1. Remove PBS completely from the sample vial.
2. Immerse samples in 0.1N iodine solution for staining on a nutator at room temperature.
3. The volume of iodine solution and staining time for different developmental stages of mouse embryo and neonate are listed in Table 1.

Note: The suggested staining time is the minimum required time to ensure full penetrance of iodine can generate contrast homogeneously throughout the entire sample. Sample can be stored in iodine solution beyond the recommended staining time after it has been hydrogel-scaffolded. If samples have not been processed, significant sample shrinkage and distortion of samples will happen during the recommended staining period, also extended staining time is needed for E18.5 embryos and neonates to reach full penetrance. Other iodine-

based solutions (ex. Lugol solution) can also be used for sample staining. However, the required staining time to reach maximum and homogenous contrasting will need to be defined by user.

Sample mounting

4. Prepare 1% (w/v) agarose in 1X PBS, pH 7.2. Microwave the solution until agarose dissolves completely in PBS.
5. Place the melted 1% agarose solution in a water bath set at 5 °C above its gelling temperature. Wait until the temperature of the agarose solution equilibrates.

Note: The gelling point of agarose listed in this protocol (Fisher Scientific, Cat# BP160) is at 37.5 °C. Hence, we set a water bath at 42.5 °C (5 °C above) and let the melted 1% agarose solution equilibrate until it reaches 42.5 °C. This ensures the agarose solution remains as liquid but won't overheat the sample during mounting. Also, once the agarose solution is aliquoted to sample mounting tube, it quickly becomes viscous, which makes mounting and positioning the sample much easier. Practice sample mounting to get familiar with the timing of agarose gelation for better sample handling.

6. The size of sample mounting tube for different stages of embryos and neonates are recommended below:
 - a. For E8.5 to E9.5 embryos, samples can be mounted in individual 0.2 ml PCR tube.
 - b. E10.5 to E13.5 samples can be mounted in individual 2 ml screw-cap microcentrifuge tube.
 - c. E14.5 and older embryos, as well as neonates can be mounted in 6 ml transport tubes.
7. Add a small amount of agarose solution into a sample mounting tube. When the agarose gradually become viscous, transfer sample into the viscous agarose solution and let sample half embedded in the agarose. At this stage, sample will remain within the viscous solution instead of sinking all the way to the bottom of the tube.
8. Use a forceps or specula to gently position the embryo to a straight, upright position (Figure 6A) before the gel solidifies completely. Add more agarose solution to cover the sample entirely.
9. Leave the sample tube at 4 °C for 15 minutes to ensure the agarose gel has fully solidified.
10. Proceed with imaging immediately.

Instrumentation set up for Bruker Skyscan 1272

11. Power up the computer.

12. Start the Skyscan 1272 Control Software to initialize the scanner.
13. Click on the X-ray icon to warm up X-ray, which will take 15 minutes.
14. Click on the grab icon to start viewing the shadow projection image detected by the scanner live on the screen (Figure 6D) for positioning the sample.
15. Align Y-axis of the sample to the center of the detectable field of view (FOV).
16. Rotate and align Y-axis of the sample to the center of the FOV again at 90 and 180 degree. If a full 360 degree data acquisition is desired, further align Y-axis of the sample at 270 degrees as well.
17. Select the proper attenuation filter to obtain the optimal dynamic range of the attenuation signal. The X-ray source peak energy voltage and current are automatically adjusted based on the attenuation filter selection (Table 2).
18. Setting up the imaging parameters according to the stage of the embryo or neonate for image acquisition (Table 3).
19. Click the start button to begin data acquisition.

Note: The time required for data acquisition depends on the settings (pixel size, rotation step, averaging, attenuation filter/X-ray energy, 180/360 degrees acquisition). Recommendations listed in Table 3 are optimized for high-throughput screening purpose, in which the criteria was set to acquire each data set at a reasonable resolution at a reasonable time. User is highly recommended to test different settings to meet their research needs.

Image reconstruction with NRecon

20. NRecon Reconstruction (Version: 1.6.9.8; Bruker) software will be used for reconstructing acquired shadow projection images. Click on the NRecon shortcut to start the software.
21. Select “Open data set for reconstruction” from the tool bar.
22. Select the first shadow projection image of the acquired data set, click “Open”.
23. The software will check and align the dataset in the background automatically while loading for processing.
24. After dataset is fully loaded, a shadow projection image acquired at degree zero of the scanned sample will appear at the main window.
25. Set the boundaries for reconstruction along the Y-axis by moving the top and bottom red bars to set the cutoff.
26. Move the green bar to a plane of interest, click “preview” button on the Reconstruction panel to generate a preview tomographic image for adjusting the reconstruction setting.

27. A preview tomography from the selected plane will be generated. And now the function tab in the Reconstruction panel will move from “Start” to “Output”.
28. Adjust the dynamic range accordingly by moving the minimum and maximum thresholds to reduce background noise and prevent over saturation of the signal.
29. Select the file output format as “TIF(16)”.
30. If the alignment of loaded dataset needs to be adjusted:
 - a. Select “Start” tab on the Reconstruction panel:
 - b. Click “Fine tuning” button.
 - c. Select “Post-alignment”, set the number of trials and parameter step, and click “Start” for testing.
 - d. Once the process is finished, select the image with the best alignment and use the corresponding post-alignment paramant for reconstruction.
 - e. Repeat the “Fine tuning” process with different parameter step if necessary.
31. Image can also be rotated through the y-axis of the sample:
 - a. Select “Setting” tab on the Reconstruction panel.
 - b. Click on the CS rotation (deg).
 - c. Enter the desired degree of rotation into the “New value” column, then click “OK” button.
 - d. Select “Start” tab on the Reconstruction panel.
 - e. Click the “preview” button to generate a rotated reconstruction.
32. To start reconstruction, select “Start” tab on the Reconstruction panel. Click “Start” button to start processing the loaded dataset.
33. Click “Add to batch” if multiple datasets are being processed. Current reconstruction job will be sent to Batch manager and appear in the panel. Repeat step 21 to add another dataset. Once all the datasets have been added, click “Start batch” button on the Batch manager panel to start processing the jobs listed.

SUPPORT PROTOCOL 2

Performing the hydrogel crosslinking with common laboratory equipment

Although the hydrogel polymerization system (Logos Biosystem) provides an all-in-one, ready-to-use solution on removing oxygen during hydrogel-tissue crosslinking, tissue-polymer crosslinking can easily be performed with the same efficiency by using common laboratory equipment. Here we provide a procedure by using vacuum desiccator and laboratory built-in benchtop vacuum to remove air from the samples (Figure 3). Samples are further purged with nitrogen before crosslinking reaction to ensure that the reaction is under

minimum influence of the oxygen to increase efficiency (Chung and Deisseroth, 2013; Wong et al., 2013b). The crosslinking reaction can then be initiated in a laboratory incubator or water bath at 37°C for 3 hours to complete the crosslinking reaction.

Materials

Nalgene™ transparent polycarbonate vacuum desiccator (Thermo Scientific™ Cat# N53110250)

Laboratory build-in benchtop vacuum

Water bath

Incubator

Industrial grade nitrogen (Airgas, size 200 cylinder, Cat# NI 200)

Oxygen removal and nitrogen purge for hydrogel polymerization

1. After samples have been immersed in either STABILITY or X-CLARITY™ buffer, transfer the sample tubes or samples plates to a vacuum desiccator.
2. Unscrew the cap completely and place it on top of each sample tube. If samples are immersed in a multi-wells plate, make sure the plate is not wrapped and sealed with parafilm.
3. Close the lid of the desiccator, connect the stopcock to a benchtop vacuum outlet via a ¼" tubing.
4. First turn on the vacuum, then open the stopcock on the desiccator to remove air for 10 minutes. During the process, the lid on the desiccator will become airtight.
5. After vacuum for 10 minutes, first close the stopcock on the desiccator, then turn off the vacuum.
6. Disconnect the vacuum tubing from the stopcock on the desiccator.
7. Connect tubing from a compressed nitrogen cylinder to the stopcock. Open the valve on the desiccator first, then turn on the compressed nitrogen.
8. Purge the samples in the desiccator with nitrogen set at 10 psi for 5 minutes.
9. After purge with nitrogen for 5 minutes, first turn off the nitrogen tank, then close the stopcock on the desiccator.
10. Open the lid of the desiccator and immediately tighten the cap on each sample tube. For samples in the multi-wells plate, seal the edge of the plate with parafilm immediately.
11. Incubate samples in a water bath or an incubator at 37 °C to initiate the crosslinking reaction for 3 hours.

REAGENTS AND SOLUTIONS

16% (w/v) paraformaldehyde stock solution

1. 16% paraformaldehyde was prepared in 1X PBS (pH 7.2) in a chemical fume hood to avoid inhalation of fumes.
2. To prepare 500 ml of 16 % paraformaldehyde, first add 300 ml of 1X PBS (pH 7.2) to a glass beaker. Microwave the buffer to bring temperature to approximately 60 °C; make sure the buffer does not boil.
3. Put the beaker on a heated stir plate in a chemical fume hood to maintain the temperature at 60 °C. Add 80 g of paraformaldehyde into the beaker while stirring.
4. Slowly add 1N NaOH dropwise with a Pasteur pipet into the solution while stirring until all the paraformaldehyde powder dissolves and the solution become clear.
5. Wait until the temperature cools down to room temperature, re-adjust the pH again to 7.2 with concentrated hydrochloric acid.
6. Adjust the final volume to 500 ml with 1X PBS, pH 7.2.
7. Filter the solution through a 0.22 um filter and aliquot into 50 ml conical tubes.
8. Store the 16% paraformaldehyde stock solution at –20 °C for up to three months.

STABILITY hydrogel solution

1. The final concentration of STABILITY hydrogel solution contains: 4% (w/vol) PFA, 4% (w/vol) acrylamide, 0.05% (w/vol) bis, 0.25% VA044 initiator, and 0.05% (w/vol) Saponin in 1X PBS.

Note: To prepare 200 ml of STABILITY hydrogel solution:

50 ml of 16% PFA solution,

20 ml of 40% acrylamide,

5 ml of 2% bis solution,

500 mg of VA044,

100 mg of Saponin

Add all the components to 100 ml 1X PBS (pH 7.2) in a glass beaker.

Stir until all the components dissolve completely. Adjust the final volume to 200 ml with 1X PBS. Keep the solution on ice if it is to be used immediately.

2. The solution can be stored at 4 °C for up to a week.

X-CLARITY™ Polymerization Initiator stock solution

1. To prepare a 25% (w/v) stock solution, add 2.5 g of the X-CLARITY™ Polymerization Initiator (Logos Biosystem, Cat# C1310X) in 10 ml of 1X PBS (pH 7.2), mix well until fully dissolved.
2. Aliquot 500 ul per 1.5 ml microtube.
3. The stock solution can be store at –20 °C for up to three months.

X-CLARITY™ hydrogel working solution

1. Add 500 ul of the X-CLARITY™ Polymerization Initiator stock solution (25%) to 50 ml of the X-CLARITY™ Hydrogel solution (Logos Biosystem, Cat# C1310X). Mix well until fully dissolved. Keep the working solution on ice.
2. The working solution can be stored at 4 °C for up to a week before use.

COMMENTARY**Background Information**

During development, embryos go through rapid and dramatic structural and morphological changes. Dysregulated development processes at any stage can lead to embryo lethality or congenital disorders. Because of the similarity in development between mice and humans as well as the high homology between mouse and human genomes, the mouse is an ideal model system for studying murine gene function in development and disease and inform on how homologous genes might function in human development and diseases (Dickinson et al., 2016). Several methods, such as high resolution episcopic microscopy (HREM), optical projection tomography (OPT), and microCT, have been used for obtain 3D volume information on studying mouse development (Sharpe et al., 2002; Wong et al., 2015; Metscher, 2009b; Wong et al., 2013b; Weninger et al., 2006; Mohun and Weninger, 2012; Hsu et al., 2016). However, each imaging method has its own strengths and limitations when applied to mouse embryo and neonate phenotyping and morphology analysis.

HREM combines histology and episcopic imaging to generate the highest resolution images of the entire 3D volumes of mouse embryos at any stage (Weninger et al., 2006; Mohun and Weninger, 2012). However, it requires an extended amount of time collecting 3D volume data by repeating the process on microtome sectioning and imaging the block face of embedded samples. Also, its applicability is restricted because there is no commercially available system for researchers who might want to adopt the technique. Optical projection tomography (OPT) has also been established to capture the tomographic information of mouse embryos via auto-fluorescence from the sample for 3D morphology and 2D virtual sections analysis (Wong et al., 2013a; Sharpe et al., 2002). To capture the projection tomography from sample's auto-fluorescence, tissues have to undergo dehydration and sample clearing process to make them optically transparent and to reduce light scattering (Sharpe et al., 2002). However, OPT imaging becomes challenging when sample size increases. Because the requirement for an extended depth of field (DOF) increases with sample size, embryos older than E15.5, as well as neonates have reduced spatial resolution. Meanwhile, the implementation of OPT imaging is also limited by lacking a commercially

available, off-the-shelf system that can cover the sample size ranging from micrometers to centimeters for high resolution imaging.

Recent development on iodine contrast microCT imaging has demonstrated that it can be easily implemented for high throughput 3D gross morphology and 2D virtual sections on studying mouse development (Wong et al., 2014; Dickinson et al., 2016; Hsu et al., 2016; Ermakova et al., 2018). With the advantages of commercial availability, high spatial resolution, and easy-to-adopt protocols, this method has become a great choice for studying mouse embryo and neonate development and 3D morphological analysis ranging from embryonic stage E8.5 to postnatal stage P4. The same protocol of sample processing and iodine contrasting can also be applied for studying abnormalities and diseases in specific mouse organs (O'Neill et al., 2017; Monsivais et al., 2019). In addition, by hydrogel scaffolding the tissues with STABILITY and X-CLARITY™ protocols, sample integrity can be preserved by minimizing sample shrinkage and decreasing the required iodine contrasting time. These standardized procedures can be easily implemented for research groups who are interested in studying mouse development from early post-implantation to early postnatal stages.

Critical Parameters

Sample preparation is important to consider when preparing for iodine contrasting microCT imaging, especially when considering the tissues present at different developmental stages. Tissues will undergo dehydration and shrink gradually when contrasted with iodine-based solutions (Degenhardt et al., 2010; Wong et al., 2012). While early post-implantation stages embryo shrink more homogeneously because of the simplicity, more complex tissue with different cellular and extracellular composition will have variable rates and ratios of shrinkage with later stage embryos and neonates. The amount and ratio of tissue shrinkage also depend on the amount of time that samples have been immersed in the iodine solution. Hence, finding the balance between the minimal required staining time and sample integrity needs to be considered before performing the imaging. For mouse embryo (E14.5 and above) and neonates, hydrogel scaffolding around the tissue forms a hydrogel-tissue hybrid that helps to reduce tissue shrinkage and reduce the staining time (Wong et al., 2013b; Hsu et al., 2016). We included two methods, STABILITY and X-CLARITY™, in this protocol that can reduce the amount of sample shrinkage. As it will take extended amount of time to generate contrast on non-stabilized embryos and neonates (days compared to weeks), we recommend implement the STABILITY protocol for preserving the embryo (E14.5 and above) and neonates. In addition, Although samples from E8.5 - E12.5 can be imaged without generation of the tissue-hydrogel structure and the anatomical structure resolved in detail (Hsu et al., 2016; Ermakova et al., 2018), the user should consider minimizing the sample shrinkage to better preserve sample integrity by process hydrogel scaffolding the embryos. This method will also better preserve the volume information and minimize shrinkage to better understand the structure changes and organ formation critical to mouse development.

In addition to sample preparation, we describe the staining process and recommended the minimum required staining time with 0.1N iodine solution for different stages of embryos

and neonates. However, other iodine based contrasting solution for soft tissue (e.g., Lugol solution and iodine potassium iodide) can be used for sample contrasting on microCT imaging (Metscher, 2009a; Wong et al., 2012). To reach the maximum contrast, we will recommend optimizing the sample staining time first with different contrasting methods for each different stage of mouse embryo and neonates in order to obtain the best signal and contrasting result. Meanwhile, although microCT imaging can easily achieve submicron resolution, it is also critical to carefully consider the balance between resolution, acquisition time per sample, and data management. Because samples size ranges from hundred microns to several centimeters during mouse development, finding the suitable parameter to be able to obtain the necessary information within the reasonable time is important. Table 1 and 3 provide the recommended parameters on imaging different stages of mouse embryos and neonates based on the Skyscan1272 (Bruker) with 0.1N iodine contrasting. We recommend users to first consider the resolution requirement for their specific research need in order to reach the optimal condition for time and data management.

Troubleshooting

Low contrast in the sample—To obtain the best results with iodine contrast microCT imaging for mouse embryos and neonates, tissues must have sufficient iodine absorption and homogeneous iodine penetrance. Low contrast or narrow dynamic range of signal usually is a direct result of insufficient concentration of iodine being absorbed by the tissue. Also, in order to obtain full iodine penetrance and homogeneous contrast, the minimum required iodine staining time increases with the size of the sample (Table 1). If iodine is not fully penetrated through the samples, the signal and contrast are much lower toward the center of the sample compared to peripheral. This issue becomes more prominent for mouse embryos at E18.5 or older, as well as neonates. A simple solution is to increase the sample immersion time in the iodine solution. It will also help by refreshing the iodine solution daily to maintain the maximum iodine concentration in the staining solution. In addition, polypropylene and polystyrene sample tubes will slowly absorb iodine and reduce the concentration of iodine in the staining solution. To ensure samples can expose to the maximum concentration of iodine during staining, we recommend using glass vials for iodine staining, especially E18.5 embryos and older, as well as neonates. It is less critical for earlier stage embryos (E8.5 to E15.5) since the amount of tissue can be saturated with iodine rapidly when immersed in fresh 0.1N iodine solution, hence these earlier stage embryos samples can be stained within multi-wells plates, 15 ml conical tube, 2ml sample tubes, or PCR tubes while still obtain sufficient contrasting results.

Iodine solution becomes colorless during sample staining—When staining samples in scintillation vials with aluminum foil liner caps, the dark brown 0.1N iodine solution will gradually become a colorless, transparent solution within several hours. Iodine (I_2) reacts to aluminum (Al) foil and form water soluble aluminum iodide (AlI_3), which interrupts the staining process as well as potentially damages the sample when the concentration of aluminum iodide rises in the solution. Avoid using scintillation cap with aluminum foil liner, select the scintillation vial caps with polyethylene cone instead.

Iodine solution becomes cloudy during sample staining—This problem is caused by carryover hydrogel solution and initiator in the samples reacting to the iodine. After polymerization, a portion of the hydrogel solution and initiator will still remain in the previously immersed sample. STABILITY and X-CLARITY™ processed samples need to be rinsed thoroughly and equilibrated in 1X PBS as suggested in Basic Protocol 1 in order to remove any unreacted hydrogel and initiator. If the hydrogel solution is not completely removed from the samples, residual STABILITY and X-CLARITY™ hydrogel solution reacts with iodine, which makes the solution cloudy and forms precipitates. These unwanted reactions between the polymer solution and iodine may damage the samples, both externally and internally. Fortunately, precipitation be noticeable immediately after iodine solution is being added to the hydrogel crosslinked sample if any hydrogel residual is still present. If the iodine solution appears to become cloudy with precipitates within 15 minutes after adding to the sample vial, remove the iodine solution immediately, and repeat the washing step listed in Basic protocol 1 until the sample is fully equilibrated with 1X PBS before staining again in the iodine solution.

Loss of contrast of soft tissue—Agarose gel (1% (w/v), prepared in sterile MilliQ water) is used as a mounting media to immobilize and position the embryos during data acquisition in this protocol. However, since iodine is only being absorbed by the sample without covalently conjugated to the tissue, it will diffuse back out into the agarose gel as soon as the sample is mounted. The more iodine diffuses out from the sample into the agarose gel, the lower the signal and contrast can be detected/resolved from the tissue. This phenomenon affects all stages of the mouse embryos and neonates but is much more prominent in the small, early post-implantation embryos between E8.5 and E12.5. Because of this reason, the volume of the agarose used for mounting sample plays a critical role of controlling the speed of iodine diffusing out of the samples. We recommend using PCR tubes with 150 μ l 1% agarose for mounting samples between E8.5 - E10.5, 2 ml sample tubes with 1 ml 1% agarose for E11.5 - E14.5, and 6 ml sample tubes with 2 ml 1% agarose for E15.5 and above to prevent rapid contrast lost after sample mounting due to diffusion.

Sample movement during data acquisition—Sample drifting during acquisition have also been observed for samples in the early post-implantation embryos between E8.5 and E12.5. Drifting is caused by the insufficient gelation of the agarose once the samples have been embedded. Although samples may only settle into the agarose several hundred micrometer more with gravity during acquisition, the amount of drifting introduces image alignment issue during image reconstruction, as well as a decrease in the image quality. Hence, the timing between sample being mounted and data acquisition become critical for early post-implantation embryos. To ensure the samples have been fully embedded and the agarose has fully solidified, simply by leaving the sample tube at 4°C for 15 minutes after sample mounting will greatly reduce this issue.

Understanding Results

Mouse embryos and neonate 3D volume data acquired by iodine contrasted microCT can be analyzed by various software packages to either analyze the 3D rendered volume for gross morphology and volume analysis, as well as sectioned virtually through different body axes

(Figure 7). Proper control samples, such as stage matched wild type or litter mate controls, are essential to compare with experimental samples to determine if there are anatomical changes and morphological differences between these groups. To understand the development and organ generation of mouse embryos, “The Atlas of Mouse Development” by M. H. Kaufman (Kaufman, 1992) is an extremely useful reference on studying the results through 2D virtual sections.

Time Considerations

To reach the best contrast, sample preparation time ranges between 3 to 10 days depending on sample size. Table 1 lists the recommended sample preparation time for embryos and neonates at different stages. For data acquisition, we list the time for acquiring then datasets at standardized resolution (Table 3). However, it is up to individual users to decide on the resolution setting and can be optimized based on user’s preference, which will have effects on the acquisition time and data size.

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Significance Statement

Iodine-contrast microCT imaging provides the advantages of commercial availability, high spatial resolution, and easy to adopt protocols for studying mouse embryo and neonate development and 3D morphological analysis. We describe two standardized and inexpensive procedures, STABILITY (for E15.5 and above) and X-CLARITY™ (younger than E14.5), by forming a hydrogel-tissue hybrid structure to minimize sample shrinkage and decrease the required iodine contrasting time for preparing mouse samples on iodine contrast microCT imaging. We also describe protocols on sample staining, mounting, and parameters for imaging different stages of mouse embryos and neonates. These standardized procedures are easy to adopt for research groups who are interested in studying mouse development from early post-implantation to early postnatal stages.

Figure 1.

Virtual sectioning of a STABILITY processed, iodine contrasted E18.5 mouse embryo imaged on microCT with a 0.5 mm aluminum attenuation filter at 11 μ m voxel size from the anterior to the posterior. The step size is 1 mm from the anterior to the posterior of the embryo. The scale bar for each panel is 2 mm.

Figure 2.
Example of a commercial stand along X-Ray microCT system (Skyscan 1272, Bruker).

Figure 3.

Example of a commercial hydrogel polymerization system (Logos Biosystem). The system contains heating blocks that can accommodate either multi-well sample plates or 50 ml conical tubes. (A) A 12-well plate and three 50 ml conical tubes were setup in the polymerization system. (B) The setting panel on the system. The crosslinking condition is set at -90 kPa and 37°C for 3 hours. (C) Alternative method with laboratory common equipment to perform the air removal process with a vacuum desiccator and benchtop built-in vacuum. (D - F) Example of a E18.5 mouse embryo after crosslinked in the STABILITY hydrogel (D-E) and removal from the embedded gel (F).

Figure 4. Comparison of E18.5 mouse embryos in (a) PBS, (b) STABILITY, and (c) X-CLARITY™ hydrogel solution after 3 hours crosslinking at 37 °C under -90 kPa vacuum (Logos Biosystem).

Figure 5. Volume difference between control and X-CLARITY™ processed mouse embryos after overnight iodine staining at E9.5 and E12.5.

Figure 6. Example of E18.5 mouse embryos mounted in (A) 1% agarose for immobilization and positioning of the sample. (B) Back projection image acquired from the detector of the iodine contrasted embryos. (C and D) Skyscan 1272 Control Software for oversize and batch scanning and imaging parameters setup.

Figure 7.
Example of volume rendering of a E18.5 mouse embryo microCT dataset and virtual sections at sagittal, transverse, and coronal axis.

Table 1.

Recommended sample fixation, hydrogel immersion, and iodine contrasting time between mouse embryo at E8.5 to neonate at P4.

Stage	4% PFA volume	Fixation time	Hydrogel volume	Hydrogel immersion	0.1N I ₂ volume	Iodine contrasting	Total
E8.5-E12.5	1 - 2 ml	Overnight	1 - 2 ml	Overnight	1 - 2 ml	Overnight	3 days
E13.5 - E15.5	5 ml	Overnight	5 ml	Overnight	5 ml	Overnight	3 days
E16.5 - E18.5	20 ml	3 days	20 ml	1 to 3 days	20 ml	3 days	7-10 days
P0 - P4	20 ml	3 days	20 ml	3 days	20 ml	3 to 7 days	9 to 13 days

Table 2.

Attenuation filter and X-Ray source energy combination on Skyscan 1272.

Attenuation Filter	Source Voltage	Source Current
No filter	50 kV	200 uA
Al 0.25mm	60 kV	166 uA
Al 0.5mm	70 kV	142 uA
Al 1mm	80 kV	125 uA
Al 0.5 + Cu 0.038mm	90 kV	111 uA
Cu 0.25mm	100 kV	100 uA

Table 3.

Suggested imaging parameter for different stages of mouse embryos and neonates.

Stage	Rotation step (Deg)	Pixel size	Attenuation filter	Acquisition time
E8.5	0.3	1.5 um	No filter	50 min
E9.5 - E10.5	0.3	3 um	No filter	50 min
E12.5	0.3	5 um	Al 0.25mm	75 min
E15.5	0.3	11 um	Al 0.5mm	75 min
E18.5 - P4	0.3	11 um	Al 0.5mm	150 - 300 min