Published in final edited form as:

Methods Mol Biol. 2015 ; 1189: 47–61. doi:10.1007/978-1-4939-1164-6_4.

Micro/Nano-Computed Tomography Technology for Quantitative Dynamic, Multi-scale Imaging of Morphogenesis

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Abstract

Tissue morphogenesis and embryonic development are dynamic events challenging to quantify, especially considering the intricate events that happen simultaneously in different locations and time. Micro-, and more recently nano-computed tomography (micro/nanoCT), has been used for the past 15 years to characterize large 3D fields of tortuous geometries at high spatial resolution. We and others have advanced micro/nanoCT imaging strategies for quantifying tissue and organ level fate changes throughout morphogenesis. Exogenous soft tissue contrast media enables visualization of vascular lumens and tissues via extravasation. Furthermore, the emergence of antigen specific tissue contrast enables direct quantitative visualization of protein and mRNA expression. Micro-CT X-ray doses appear to be non-embryotoxic, enabling longitudinal imaging studies in live embryos. In this paper we present established soft tissue contrast protocols for obtaining high quality micro/nanoCT images and the image processing techniques useful for quantifying anatomical and physiological information from the datasets.

1.0 Introduction

Growth, differentiation, and organization represent the fundamental stages of embryogenesis (Dehaan and Ebert 1964). Heterogeneous patterning of multiple cell types with complex orchestration has necessitated the development of new imaging strategies for capturing this dynamic process. Parallel advancements in experimental and imaging technologies have enabled dynamic, quantitative studies of morphogenesis and dysmorphogenesis. Three dimensional imaging modalities offer unique insights and provide quantitative information on the dynamic tissue fate changes during development.

Micro/nano-computed tomography (micro/nanoCT) has been used for the past 15 years to characterize tortuous spatial geometries at high resolutions (Butcher, Sedmera et al. 2007). A gamma source emits high powered x-ray energy whose attenuation through the sample is registered by a gamma camera opposite the emitter relative to a bone standard (Gregg and Butcher 2012). Through a back projection analysis from 360° discrete angle measurements, the image is fully registered into a series of planar slices which can be interpolated into a three dimensional reconstruction (Butcher, Sedmera et al. 2007). Scans lasting a few

minutes or less can achieve resolution at 50-25 μ m (Kim, Min et al. 2011) but longer exposure times can yield resolution into the sub-micron range (Metscher 2009).

Micro/nanoCT has become an important imaging technology for dynamic and quantitative imaging of embryonic development. Johnson and colleagues imaged transgenic mouse embryos using microCT for virtual histology. With an 8 μ m scan of wild type mice stained with osmium tetroxide, virtual histology from the microCT scan is compared to that of paraffin histology of E11.5 embryos (Figure 1, (Johnson, Hansen et al. 2006)). Using a 27 μ m scan and osmium tetroxide staining, segmentation and screening of developmental defects found in the rostral neural tube of *Pax3:Fkhr* E11.5 transgenic mouse embryos were seen with microCT imaging (Johnson, Hansen et al. 2006). Through virtual histology segmentation, the cephalic forebrain, midbrain, and hindbrain vesicles, the heart wall and cardiac ventricles, and the liver were visualized (Johnson, Hansen et al. 2006). Furthermore, the incomplete neural crest closure, overgrowth of the mesenchyme and hypotrophy of the telencephalic vesicles is seen with the 27 μ m microCT scan. Furthermore, Degenhardt and colleagues used microCT to image *PlexinD1* mutant mouse embryos at 16 μ m resolution for visualizing cardiac defects stained with Lugol's solution (Degenhardt, Wright et al. 2010). E17.5 *PlexinD1* mutant embryos were compared to wild type embryos and multi-planar reconstructions were used to determine the planes that best reveal the defects, showing intracardiac and extracardiac defects that are clearly seen with the microCT scans (Figure 1, (Degenhardt, Wright et al. 2010)).

In HH24 chick embryos, Metscher and colleagues compared different soft tissue staining techniques imaged at 12 μ m resolution (Figure 2, (Metscher 2009)). Butcher et al scanned embryonic chick hearts from HH15-HH36 perfused with Microfil™, a radiopaque casting polymer, through microinjection. The hearts were scanned at 10.5 μ m resolution and three dimensional volumetric reconstructions were rendered and compared to serial reconstructions and scanning electron micrographs. Volume changes of the atria, ventricles, outflow tract, and atrioventricular canal were quantified throughout development. The general microinjection setup and microCT volume rendering as compared to the serial sections and scanning electron micrograph are given in Figure 3 (Butcher, Sedmera et al. 2007). Kim and colleagues stained chick embryos with osmium tetroxide and characterized organ development with isosurface three dimensional renderings of chicks spanning day 4-10 of embryogenesis (Figure 3, (Kim, Min et al. 2011)).

Using the high resolution capabilities in nanoCT, Metscher et al demonstrated scan resolutions of 0.97 μ m of the developing chick embryo stained with Lugol's solution where neural crest cells can be seen (Figure 4, (Metscher 2009)). Extending past general soft tissue staining, Metscher and colleagues have demonstrated antigen-specific tissue staining with whole mount imaging of antibody probes with metal-based immunodetection (Metscher and Muller 2011). In Figure 4, antigen specific immunostaining for alpha myosin heavy chain (aMHC) and type II collagen distribution in the developing limb is given. Henning and colleagues demonstrated the first in vivo live embryonic study using microCT imaging. Henning et al quantified the level of contrast, biodistribution and organ volume of developing chick embryos (Figure 5, (Henning, Jiang et al. 2011)). Furthermore, Henning and colleagues demonstrated that radiation from the scan up to 798mGray did produce any

morphological defects up to day 10 of chick development when the experiment was ended (Henning, Jiang et al. 2011). Visipaque™ (VP) and Omnipaque™ (OP) were used as exogenous soft tissue contrast. OP was found to be embryotoxic within 24 hours post-injection due to being hyperosmotic whereas iso-osmotic VP was non-toxic up to day 10 of development (Henning, Jiang et al. 2011). Additionally, VP produced 1060 Hounsfield units of contrast with a 50µm resolution microCT scan (Henning, Jiang et al. 2011).

Micro/nanoCT imaging is a powerful tool for quantifying dynamic anatomical changes throughout development and it enables physiological changes to be measured as well. Using the image data, three dimensional models can be generated and used for computational analysis and simulation of physiological events. Yalcin and colleagues analyzed the hemodynamic patterning of the atrioventricular canal in the embryonic chick using three dimensional data obtained by microCT and flow parameters acquired from ultrasound imaging. The averaged wall shear stress was found at early, peak, and late stages of the cardiac cycle characterizing the inflow and outflow patterns seen in development (Figure 5, (Yalcin, Shekhar et al. 2011)).

Micro/nanoCT imaging is a powerful tool for studying embryonic development and quantifying anatomical and physiological changes. Through the wide variety of tissue staining techniques, high contrast, high resolution multi-scale three dimensional images are easily attained and with the emerging antigen-specific staining, localized expression changes can be differentiated and quantified. Furthermore, with the advancement into live embryonic imaging, longitudinal embryonic studies have become a tangible reality. In this paper we introduce some of the most common protocols for imaging with micro/nanoCT and the image processing techniques needed to quantify the information observed within the image.

2.0 Materials

2.1 General Soft Tissue Contrast Agents

The most prominent used methods for non-specific embryonic soft tissue contrast is given below. This information is summarized in Table 1.

2.1.1 Lugol's Solution

1. 1% weight/volume of elemental iodine (I₂, Sigma)
2. 2% potassium iodide in water (KI, Sigma)

2.1.2 Phosphotungstic Acid (PTA)

1. 1% weight/volume of phosphotungstic acid (Mallinckrodt Stains) in water
2. Absolute ethanol

2.1.3 Osmium Tetroxide

1. 1-2% osmium tetroxide in phosphate buffer (OsO₄)

2.1.4 Gallocyanin Chromalum

1. 5% weight/volume in water of gallyocyanin-chromalum (Chroma 1A)

2.1.5 Microfil TM

1. Standard petri dish
2. Earl's Balanced Salt Solution (EBSS, Sigma)
3. Microinjection protocol (see section 2.3)
4. Polymerizing contrast agent (Microfil, Flow-Tech, Inc.)

2.1.6 Omipaque™

1. Omnipaque is administered to a live specimen but it is lethal within 24 hours making it ineffective for longitudinal studies (Henning, Jiang et al. 2011)
2. Omnipaque™ (iohexol, GE Healthcare) with a concentration of 350mg/mL of iodine

2.1.7 Visipaque™

1. Visipaque is administered to a live specimen and has shown to be nonembryotoxic (Henning, Jiang et al. 2011)
2. Visipaque™ (iodixanol, GE Healthcare) with a concentration of 320mg/mL of iodine

2.2 Antigen-Specific Contrast

2.2.1 Enzyme Metallography Immunostaining (adapted from (Metscher and Muller 2011))

1. A primary antibody is chosen for immunostaining
2. Secondary antibody for immunostaining (goat anti-mouse IgG HRP conjugate, Invitrogen)
3. Glyoxal-based fixative (Shandon Glyco-Fixx, ThermoScientific)
4. 100% methanol
5. Dimethylsulfoxide
6. Hydrogen peroxide
7. Solution of 100mM maleic acid, 150mM NaCl, 0.1% Triton X-100 pH 7.4 (MABT)
8. Blocking solution of MABT with 0.1% saponin, 10% sheep serum, 0.5% Roche Blocking Reagent, DMSO
9. Nanoprobes EnzMet 6010 Enzyme Metallography Kit (Yaphank, NY)
 - a. The EnzMet kit from Nanoprobes is comprised of 3 different solutions based on the chemical reduction reaction in peroxidase to reduces silver ions (Ag⁺)

to insoluble metallic silver (Ag₀), precipitating in the immediate vicinity of the enzyme conjugate

- b. Using a peroxidase-conjugated secondary antibody and following the EnzMet kit instructions, the silver precipitate acts as the tissue specific contrast agent for CT imaging of the specimen

2.3 MicroInjection

1. Borosilicate glass capillary tubes (Sutter Instrument Co, OD 1.0mm, ID 0.75mm)
2. Small diameter silicone tubing (VWR)
3. 3mL plastic syringe (BD)
4. Laboratory stand
5. Micromanipulator

2.4 Micro/NanoCT Imaging

1. eXplore CT series scanners (GE Healthcare)
2. Xradia CT series scanners (Xradia Inc.)

2.5 Image Filtering, Rendering, and Analysis

1. Useful Software Platforms for Image Processing
 - a. OsiriX (Rosset et al 2004)
 - b. MicroView (GE Healthcare)
 - c. ImageJ (National Institutes of Health)
 - d. GAMBIT (ANSYS Inc.)

3.0 Methods

3.1 General Soft Tissue Contrast Agents

3.1.1 Lugol's Solution

1. In-dionized water, combine the iodine metal and the potassium iodide and then aliquot out the necessary amount needed for the sample.
2. Prior to staining the sample, the solution should be diluted to 10% of its initial concentration with de-ionized water.
3. Using samples that have been stored in aqueous solution, the specimen is stained for a minimum of 30 minutes to overnight depending on the size and density of the sample being stained.
4. Once the staining process is complete, the sample should be washed with water prior to imaging and once the imaging is complete the sample is dehydrated in ethanol and stored.

5. Lugol's solution is stable for several months.

3.1.2 Phosphotungstic Acid

1. The stock solution is a 1% weight/volume solution of phosphotungstic acid in water.
2. For staining samples, mix 30% of phosphotungstic acid stock solution with 70% absolute ethanol.
3. For this staining protocol, samples should be dehydrated in 7-% ethanol prior to staining. For staining the specimens, suspend the sample in the above solution for 2 hours to overnight depending on the size and density of the sample.
4. Prior to imaging, wash the samples with 70% ethanol and scan in 70-100% ethanol.
5. This solution is stable for several months.

3.1.3 Osmium Tetroxide

1. Using samples that are in an aqueous solution, stain the sample for 1-2 hours with 1-2% OsO₄ in phosphate buffer
2. Once the staining is complete, the sample should be washed in phosphate buffer and dehydrated in ethanol for storage.

3.1.4 Gallocyanin Chromalum

1. Working with 5% weight/volume gallocyanin-chromalum in water, the solution should be boiled to dissolve all of the gallocyanin-chromalum generating the working contrast agent.
2. Once the gallocyanin-chromalum has been dissolved, allow the solution to cool and then filter the solution.
3. The samples should start in an aqueous solution medium prior to staining and before exposing the samples to the gallocyanin-chromalum stain they should be washed in water.
4. The optimal contrast, the samples should be stained overnight and then re-washed with water prior to imaging.
5. Once imaging has been completed, the samples can be dehydrated in ethanol and stored.
6. The solution is stable for 1-2 weeks

3.1.5 Microfil™

1. Begin by placing the sample in a culture dish with a small amount of buffered saline
2. Microfil is perfused at a ratio of 80:15:5 diluent:agent:catalyst (Microfil, Flow-Tech Inc.) in the desired location for imaging via microinjection introduced in section 3.3.

3. Perfusion requires approximately 10 minutes before it is complete.
4. After perfusion, embryos should be fixed in whichever method the investigator prefers and is then stored.

3.1.6 Omnipaque™

1. Omnipaque™ is microinjected into the specimen via the protocol introduced in section 3.3.
2. The specimen should be imaged immediately post injection, especially if the cardiovascular system is of particular interest in the study due to the quick extravasation of OP from the vessels.

3.1.7 Visipaque™

1. Visipaque™ is microinjected into the specimen via the protocol introduced in section 3.3.
2. The specimen should be imaged immediately post injection, especially if the cardiovascular system is of particular interest in the study due to the quick extravasation of VP from the vessels.
3. Previous studies demonstrate the biodistribution kinetics of Visipaque and the large organ systems that the contrast agent resides in post injection (Henning, Jiang et al. 2011).

3.2 Antigen-Specific Contrast

3.2.1 Enzyme Metallography Immunostaining (adapted from (Metscher and Muller 2011))

1. The embryos should be fixed in the glyoxal-based fixative. After fixation the sample is rinsed with 100% methanol and stored.
2. Incubate the embryos in a solution of methanol, DMSO, and hydrogen peroxide for 12 hours.
3. The specimen should be washed twice in 50% methanol and rehydrated through a methanol series to MABT solution
4. Wash the samples in the MABT solution (described in section 2.2) + 0.1% saponin for 10 minutes followed by blocking the sample at room temperature with the blocking solution (given in section 2.2).
5. Incubate the sample with the primary antibody in a ratio of 1:500 in blocking solution for 2-3 days and then wash the sample in the blocking solution overnight.
6. Incubate the sample with the secondary antibody in a ratio of 1:500 blocking solution for 2-3 days.
7. Once primary and secondary antibody steps are complete, the sample should be washed several times in the MABT solution with a final MABT wash overnight.

8. Postfix the specimen in 10% formalin in MABT for 20 minutes and then wash in double distilled water 3 times for 10 minutes during each wash.
9. Change the solution that the samples are in to a solution of 0.1% Triton X-100 in distilled water.
10. Transfer the immunolabeled specimens in 0.1% Triton X-100 to clean tubes and remove the Triton X-100 solution.
11. Once all steps to this point have been completed, the reagents supplied by Nanoprobes Inc. EnxMet 6010 kit will be used for the remaining procedure.
 - a. 300-400uL of Solution A should be added and mixed gently for 4 minutes
 - b. Repeat the above step for Solution B and Solution C supplied by Nanoprobes Inc.
 - c. Once all reagents have been added and thoroughly mixed, monitor the specimen under a dissecting microscope for 20-40 minutes with gentle agitation
 - d. Change to the stop solution just as the solution begins to appear gray and then rinse in distilled water
12. Complete a methanol series by first washing the samples in 75% methanol followed by 100% methanol.

3.3 MicroInjection

1. Pull borosilicate glass capillary tubes into microneedles.
2. The needles should be cut to the desired diameter and beveled to 45° with a microforge.
3. The assembly of the microinjection apparatus is as follows:
 - a. The silicone tubing is attached to the 3mL syringe via a pipette tip
 - b. The agent being injected is loaded into the silicon tubing and the needle is attached to the end of the tubing
 - c. A gravity-driven pressure gradient is established with the micromanipulator attached to a laboratory stand
 - d. The soft tissues of the embryo are visualized through a dissection microscope
 - e. The needle is positioned into the vessel of the embryo and pressure through the syringe is applied, injecting the agent into the specimen.

3.4 Micro/NanoCT Imaging

1. For live embryonic imaging, a scanner that only rotates the gantry and not the sample is necessary, this type of scan is typical for a microCT scanner such as the GE eXplore but not for the Xradia scanner.

2. Resolution of the image is largely dependent on the field of view, for live studies 25 μ m voxels have been reported (Kim et al 2011) but for fixed embryonic studies resolutions have been reported to be as small as 0.97 μ m voxels on an Xradia imaging system (Metscher et al 2008).

3.5 Image Filtering, Rendering, and Analysis

3.5.1 Image Filtering

- a. The first step for image processing is to determine the structure of interest in the image. Contrast levels can be changed in OsiriX along with basic filtering techniques such as thresholding for segmenting out structures based on defined contrast levels. Additionally, ImageJ is easy to use and open source for basic filtering techniques (ie. thresholding) and the image file type is readily changed in ImageJ which is convenient for opening the image in a separate software platforms.
- b. Furthermore the level of contrast, measured in Hounsfield units, can be analyzed through virtual cross sections within the image and displayed with a contrast vs. spatial location plot to understand the contrast enhancement of the desired anatomical structure. MicroView easily plots this information through the use of its line tool feature. Absolute Hounsfield unit measurements are indicative of relative contrast biodistribution at the given time when the image was taken
- c. Image data sets, either in TIF or DICOM format, can be easily reconstructed through volumetric rendering, maximum intensity projections, and multi-planar reconstruction through OsiriX
- d. Region growing algorithms are established through user defined seed points positioned in the region of interest (ROI), typically a single anatomical segment or organ. The local gradient magnitude is used to find the standard deviation to segment out the single structure of interest. This is done effectively by setting pixel values in the ROI to zero and then propagating this negative space based on the user defined seed point through the Z plane (Kim, Min et al. 2011)

3.5.2 Rendering

- a. Image data sets, either in TIF or DICOM format, can be easily reconstructed through volumetric rendering, maximum intensity projections, and multi-planar reconstruction, most easily achieved in OsiriX.
- b. Region growing algorithms are established through user defined seed points positioned in the region of interest (ROI), typically a single anatomical segment or organ. The local gradient magnitude is used to find the standard deviation to segment out the single structure of interest. This is done effectively by setting pixel values in the ROI to zero and then propagating this negative space based on the user defined seed point through the Z plane (Kim, Min et al. 2011). Segments from the image based on region growing or user defined regions of interest can be reconstructed through volumetric renderings, maximum intensity projections, and multi-planar reconstructions.

3.5.3 Analysis

- a. For anatomical analysis, renderings obtained from the images can be used to make basic measurements such as surface area and volume. OsiriX is an easy software to make these measurements in. Additionally, for simple measurements ImageJ is ideal for user defined line segmenting where small measurements can be made.
- b. For physiological simulations, geometries obtained from the renderings described in the section above can be imported into computational analysis software such as GAMBIT and meshed optimizing tetrahedral elements for analysis by varying the surface and volume.
- c. ANSYS 12 FLUENT is a well known computational fluid dynamics solver designed for second-order double precision 3D pressure driven complex flows based on the meshed geometries from GAMBIT. From user defined flow conditions, fluid simulations can be performed based on the anatomical geometry from the microCT image. It is reasonable to have 30-50 time steps and 1,000 iterations of each simulation for analyzing regions of flow (Yalcin, Shekhar et al. 2011).

4.0 Notes

1. General soft tissue contrast agents all vary in the time for staining and if the sample should be in an aqueous or alcohol medium, be sure to make note of that when considering which contrast agent is the most appropriate for the given study.
2. Osmium tetroxide produces excellent contrast but it is toxic and disposal can be expensive.
3. Be aware of how dense your embryonic sample is based on its age in development and make sure to adjust the staining time accordingly, err on the side of longer rather than shorter staining times.
4. The whole embryo staining options will begin to leach out of the tissues once the staining procedure has ended; therefore, the sample should be imaged as soon as possible for maximum contrast.
5. Microfil™, Omnipaque™, and Visipaque™ contrast agents are injected into the specimen, microinjections are time consuming to master and these stains take additional considerations given this requirement.
6. The antigen-specific staining works on the principle of the secondary antibody reacting through a reduction reaction of the silver ions to insoluble metallic silver which acts as the contrast agent. This reaction happens when a peroxidase is present; therefore, must be inherent to the secondary antibody otherwise the reaction will not take place and the silver precipitation will not occur.
7. Many different software platforms exist for image processing and the few mentioned in this paper is not an exhaustive list but simple suggestions based on experience, several different software options are available and most of the time it is simply based on accessibility and preference to the investigator.

8. OsiriX is capable of many different image processing tasks and has proved itself to be a valuable tool but it is only available for Macintosh computers, there is no PC alternative for OsiriX.
9. ImageJ is an initial place to start with image processing, thresholding is easy in ImageJ and slices of the image stack can be eliminated which can significantly reduce the file size.
10. For the input parameters in fluid simulation studies, if literature values are not available then Doppler ultrasound imaging produces excellent flow information that can be used in the simulations.

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Figure 1.

(A) Comparison of paraffin and virtual histology of E11.5 mouse embryos scanned at 8um with isosurface renderings in the top row, traditional histology sections from a littermate in the middle row, and the virtual histology via microCT in the bottom row (Johnson, Hansen et al. 2006). (B) Multi-planar reconstructions of E17.5 wild type mice and PlexinD1 mutant mice in the black and white images of the top and bottom rows respectively. The red images illustrate volumetric reconstructions of the wild type (top row) and mutant mice (bottom row) demonstrating the malformation of the aorta and pulmonary artery (Degenhardt, Wright et al. 2010).

Scale Bars: (A) 400µm (B) 500µm

Figure 2.

Comparison of common contrast stains denoted on the image with resolution ranging from 4.6 μm – 5.1 μm (Metscher 2009).

Scale bar is 500 μm

Figure 3.

(A) Micro-injection set-up (Butcher, Sedmera et al. 2007) (B) Isosurface renderings of day 4,7,10 (left to right) chick embryos with colorized segments showing the cranial neural crest (light blue), eye (purple), limb (green), cardiac muscle (brown), and conotruncal cavity (dark blue) (Kim, Min et al. 2011), (C) microCT image of an HH30 chick heart (left) as compared to the serial reconstruction (middle), and the scanning electron micrograph (right) (Butcher, Sedmera et al. 2007).

Scale bar is 1mm.

Figure 4.

(A) Volumetric reconstruction from a microCT image of limb cartilage patterning over development at HH27,29,31 of the chick embryo with antigen-specific staining of type II collagen at resolutions between 3.8-4.8 μ m (Metscher and Muller 2011). The arrow indicates the vestigial anlage of digit 5 in the forelimb. Scale bar is 500 μ m. (B) Alpha myosin heavy chain antigen-specific staining in the chick embryo. (C) High magnification image of a stage 12 chick embryo with resolution at 0.97 μ m where neural crest cells are visualized (Metscher 2009).

Scale bar is 100 μ m.

Figure 5.

(A) 2D and 3D rendered 50µm microCT images of live day 3 embryonic chicks injected with VP (Henning, Jiang et al. 2011). (B) Quantitative volumetric analysis of day 4 chick embryos imaged at 50µm (Henning, Jiang et al. 2011). Dark blue represents the hindbrain, light blue is the forebrain, green in the eye, and purple is the heart. (C) 3D hemodynamic environment of the atrioventricular canal in a HH27 chick embryo (Yalcin, Shekhar et al. 2011).

Scale bar is 200µm.

Table 1

General Exogenous Soft Tissue Contrast for CT Imaging (adapted from (Metscher 2009))

Contrast Stain	Lugol's Solution	Phosphotungstic Acid	Osmium Tetroxide	Gallocyanin-Chromalum	Microfil™	Omnipaque™	Visipaque™
Stain Solution	1% I ₂ + 2% KI	1% PTA in H ₂ O Mix 30% PTA + 70% absolute EtOH	1-2% OsO ₄ in phosphate buffer	5% dissolved in billing water, cooled, and filtered	Mix in a ratio of 80:15:5 diluent:agent:catalyst	N/A	N/A
Staining Protocol	Stain 30 minutes to overnight Wash in H ₂ O, dehydrate in EtOH	Stain 2hr to overnight Wash in EtOH Scan in 70-100% EtOH	Stain 1-2 hours Wash in phosphate buffer Dehydrate in EtOH	Stain overnight, wash in water, and dehydrate in EtOH	Inject into structure being imaged, polymerizes in approximately 10 minutes	Microinject into vascular system of specimen, specimen is alive for imaging	Microinject into vascular system of specimen, specimen is alive for imaging
Advantages	Rapid, deep penetration, cheap, robust, stock solution stable for months	High levels of contrast, stock solution is stable for months, sharp tissue differentiation	Combines with extracellular matrix, high contrast	Allows for cell densities and individual cells to be visualized	Imaging negative spaces, remains isolated once polymerized, high level of contrast	Allows for short term live imaging	Allows for long term live imaging
Limitations	Overstain some mineralized tissues, intensity can vary	Slow tissue penetration (few millimeters)	Toxic with expensive disposal, limited penetration, cannot be used with alcohol-stored tissues	Overall low level of contrast	Complicated staining protocol, requires microinjections	Embryotoxic within 24 hours, requires microinjection, low residence time	Requires microinjection, low residence time