Surface Imaging Microscopy, An Automated Method for Visualizing Whole Embryo Samples in Three Dimensions at High Resolution

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Summary

Modern biology is faced with the challenge of understanding the specification, generation, and maintenance of structures ranging from cells and tissues to organs and organisms. By acquiring images directly from the block face of an embedded sample, surface imaging microscopy (SIM) generates high resolution volumetric images of biological specimens across all of these scales.

Surface imaging microscopy expands our range of imaging tools by generating three-dimensional reconstructions of embryo samples at high resolution and high contrast. SIM image quality is not limited by depth or the optical properties of overlying tissue, and intrinsic or extrinsic alignment markers are not required for volume reconstruction. These volumes are highly isotropic, enabling them to be virtually sectioned in any direction without loss of image quality. Surface imaging microscopy provided a more accurate three-dimensional representation of a chick embryo than confocal microscopy of the same sample. SIM offers excellent imaging of embryos from three major vertebrate systems in developmental biology: mouse, chicken, and frog. Immediate applications of this technology are in visualizing and understanding complex morphogenetic events and in making detailed comparisons between normal and genetically modified embryos.

Introduction

Motivation

We are interested in understanding the way in which cellular function and behavior generates structures of high complexity, such as tissues and organs. The most logical structure around which to organize and synthesize these different categories of information is the three dimensional anatomy of the specimen. In short, we must know both where and when an event occurs before we can understand how. Such an effort will require imaging techniques spanning several orders of magnitude in spatial and temporal resolution. Current imaging techniques provide tools for high resolution imaging of small volumes or low resolution imaging of large volumes. A technique is needed to bridge this gap. Surface imaging microscopy meets this need.

Properties of the Basic Technique

Surface imaging microscopy (SIM) is an automated imaging technique that captures fluorescence images from the freshly cut surface of an opaque polymer block. The microscope is comprised of an integrated microtome and widefield fluorescence microscope, with a computer controlled translation stage that holds the embedded sample, draws it over a diamond knife, and returns the sample to the field of view of the objective lens. Images can be collected into one, two, or three independent fluorescent channels, and the signal can be either the inherent autofluorescence of the tissue or extrinsic contrast supplied by a fluorescent dye.

The depth of field in a SIM image is set by the amount of opacifier added to the embedding polymer. This amount can be empirically tuned to closely match the in-plane resolution of the image through the range of magnification of 2x-40x microscope objectives, corresponding to 8.8–0.4 micron resolution and sample sizes from 8–0.5 millimeters. The resulting SIM dataset is nearly isotropic, as the in-plane resolution of the microscope objective lens is matched to the thickness of the sections removed and to the depth of field of the image as determined by the penetration depth of light into the sample. The field of view of the microscope objective determines the in-plane sample size, but there is no inherent limit to the total depth of the specimen that can be sampled. The immediate output of SIM is a registered series of two-dimensional images, which are then computationally reconstructed into a three-dimensional volume. The realignment accuracy of the stage is sufficient that the misregistration between subsequent images is sub-resolution and no post-hoc realignment is necessary. Figure 5-1 schematically depicts a surface imaging microscope.

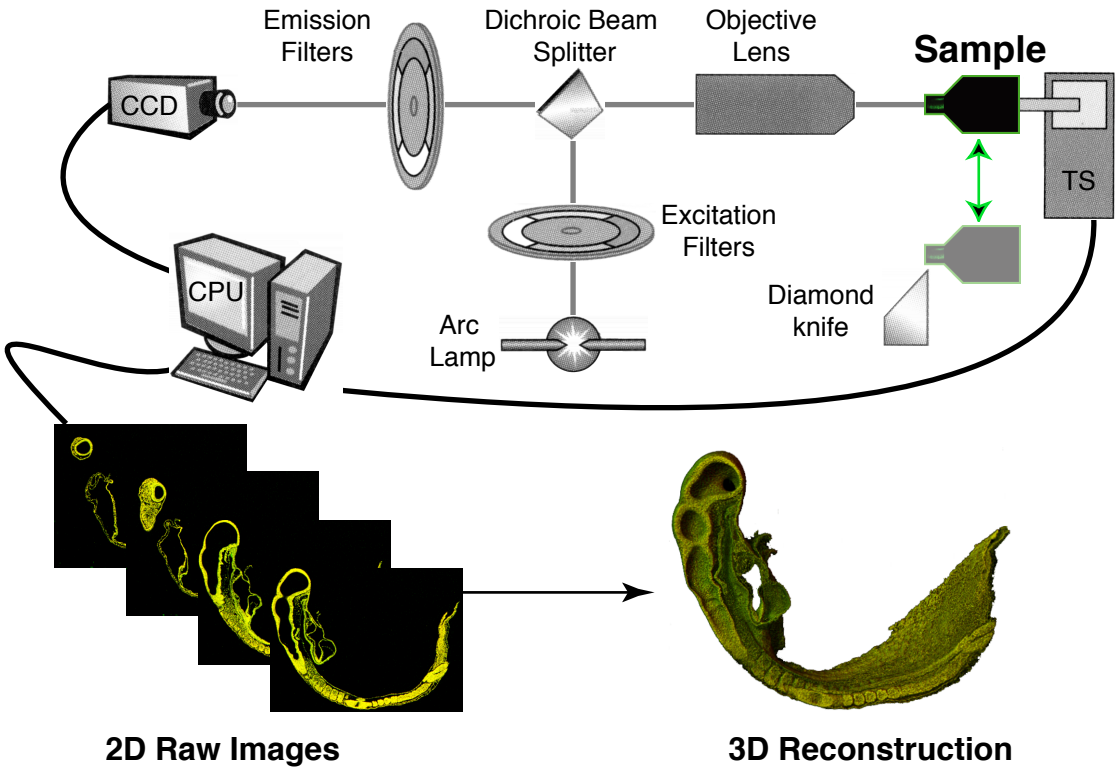
Results and Discussion

Quality of 2D Images

Figure 5-2 depicts three orthogonal views through a typical SIM dataset of a chick embryo (stained with Resolution Standard Stain). The main panel is the raw x-y image collected from the block surface. The x-y view demonstrates that the raw images of a SIM dataset have high contrast, high resolution, and excellent tissue preservation. Additionally the embryo is clearly distinct from the

Figure 5-1, Description of the Basic Technique: Schematic depiction of surface imaging microscopy (SIM). A fluorescently labeled specimen is fixed, labeled, dehydrated, infiltrated, and embedded in a black polymer, then loaded onto a motorized translation stage (TS). A computer (CPU) controls synchronizes the movement of the sample over a diamond knife, removing a thin section of material and returning the sample to the field of view of an objective lens. An image of the surface of the block is collected by a charge-coupled device (CCD) camera and the process repeats. This aligned series of two-dimensional images can then be computationally reassembled into a three-dimensional volume in standard image processing applications.

Figure 5-2, Evaluation of Image and Dataset: Two-dimensional data resulting from a SIM dataset of a fluorescently labeled chick trunk, stained with Resolution Standard Stain (Resolution Sciences Corporation, Corte Madera, CA). Anterior is to the left. Note in particular the similarity in contrast, resolution, and level of detail in the three views. The raw x-y image collected by the CCD camera corresponds closely to a frontal section through the embryo. This image is a representative single section from the dataset. The red and green lines mark the axial levels at which the orthogonal views were extracted. The x-z view is a computationally reconstructed sagittal view of the dataset, highlighting the developmental progression of somites as a function of distance along the anterior-posterior axis. The y-z view is a computationally reconstructed transverse view through the dataset, highlighting the neural tube, notochord, and paired somites. Scale bar = 100 microns.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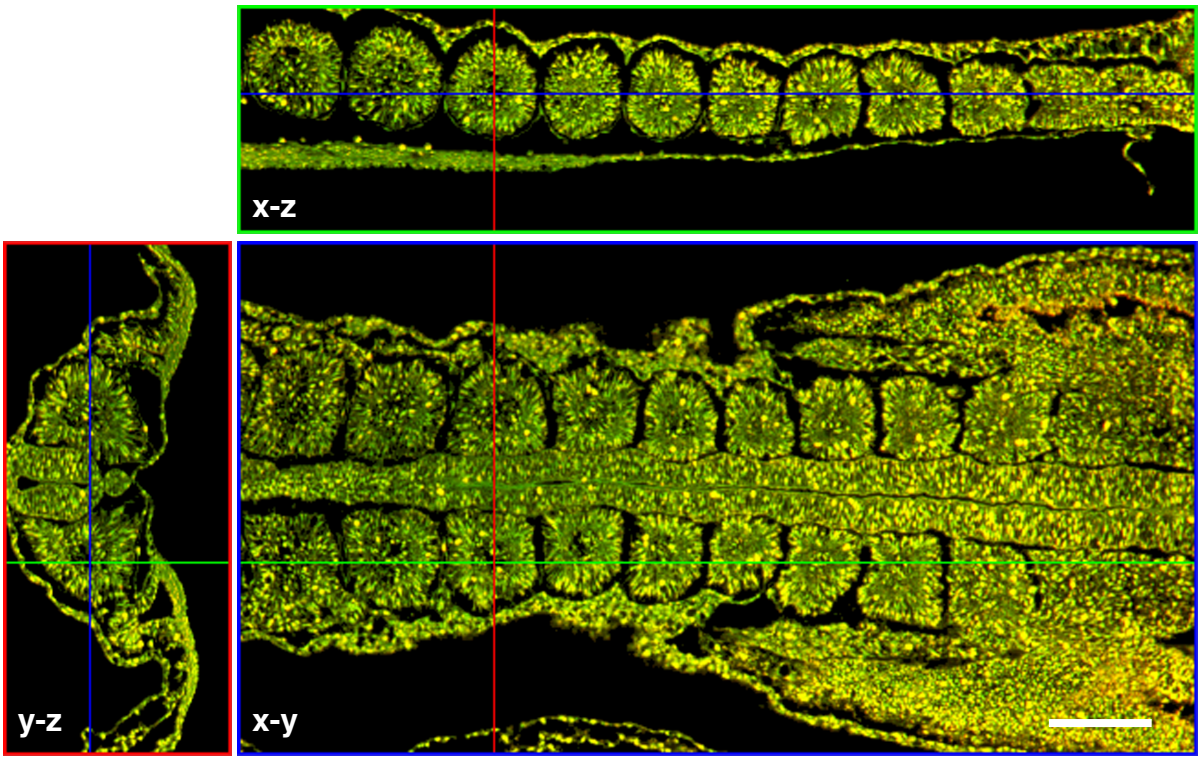


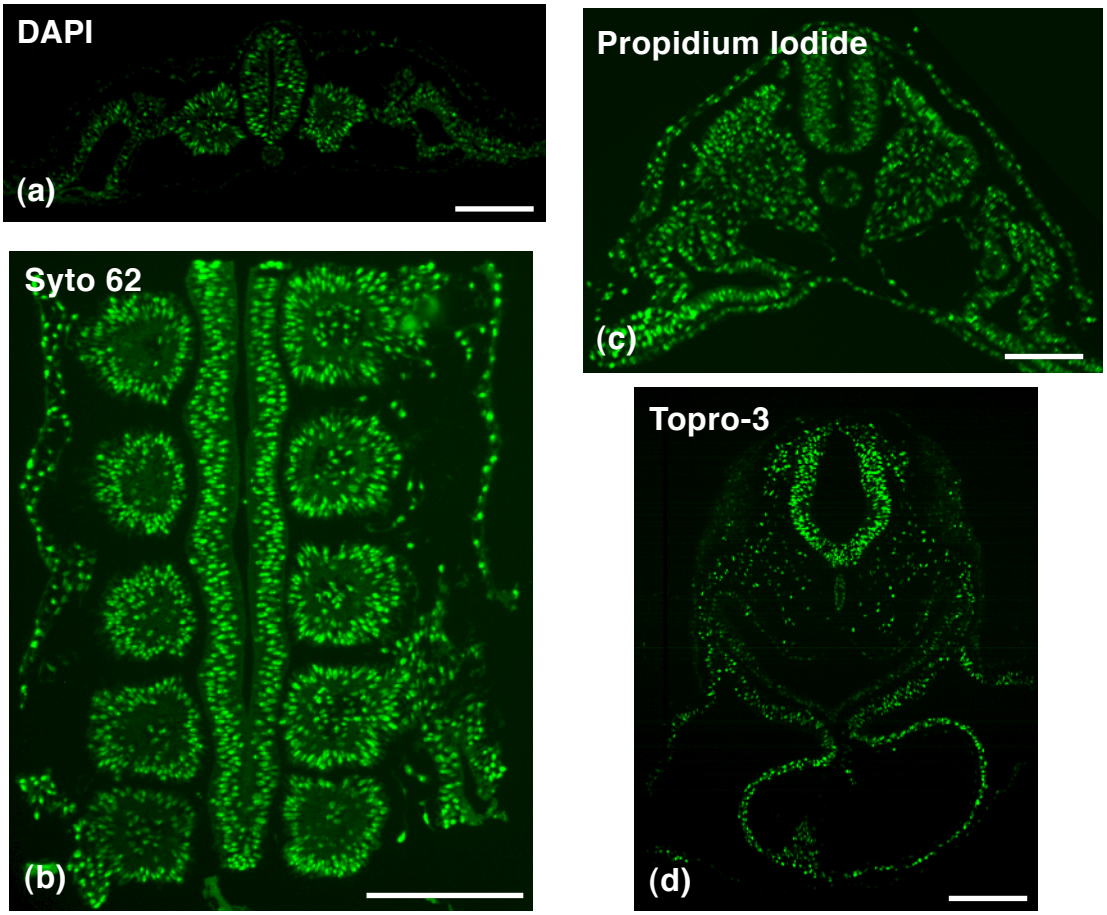
Bar = 100 microns

surrounding block, greatly facilitating three-dimensional reconstructions. It is also evident from the x-y view that there is minimal knife chatter in the image, and that SIM captures a wide field of view at cellular resolution. We have collected images of similar quality using tissue autofluorescence, DAPI, propidium iodide, Syto 62, and Topro-3 (Molecular Probes, Eugene, Oregon); Figure 5-3 shows 2D images acquired with each of these dyes. These dyes range in emission wavelength from the near UV through the near-IR and demonstrate that the embedding polymer and the imaging system are sensitive to the entire visible spectrum.

Quality of Datasets

Orthogonal sections through the dataset are necessary to critically assess many aspects of the quality of SIM datasets; these sections reveal time and depth dependent variations in image quality. Figure 5-2 presents x-z and y-z sections through the same dataset, at the levels indicated by the colored lines. Changes in fluorescence excitation intensity or camera efficiency over the course of the imaging would show up in the x-z or y-z sections as lines that were aberrantly brighter or darker than adjacent sections. This artifact is not typically present in SIM datasets. Misregistration of the blockface during imaging would cause smooth curves within features of the dataset to appear jagged, as structures that were adjacent in the sample are mistakenly separated in the resulting dataset. The contiguous outlines of individual cells and the smooth outlines defining tissue blocks and cavities argues that any registration errors are sub-resolution. No

Figure 5-3: SIM Images of Various Nuclear Dyes: A: Transverse section through the trunk of a chick embryo, labeled with DAPI and imaged using SIM. B: Frontal section through the trunk of a chick embryo, labeled with Syto 62, and imaged using SIM. C: Transverse section through a chick trunk, labeled with propidium iodide, and imaged using SIM. D: Transverse section through rhombomere four of a chick hindbrain, labeled with Topro-3, and imaged using SIM.



Bar = 50 microns

alignment algorithms have been used to process this dataset; the registration evident in Figure 5-2 is indicative of the alignment of the raw data.

SIM relies on staining samples in whole mount, then imaging thin optical sections of the surface of the sample. As a result the dye needs to penetrate adequately in the whole sample and then be sufficiently bright in the thin optical section. Inconsistencies in dye penetration would show in the x-z and y-z images as changes in color or intensity through structures of similar cellular composition, such as epithelial somites. Figure 5-2 is clear evidence that our labeling techniques yield uniformly stained samples and that SIM has the sensitivity to detect the fluorescent signal from thin optical sections.

A major technical advantage of SIM over previous blockface imaging efforts [1-4] is the isotropicity of the resulting 3D datasets. The axial resolution of any three dimensional imaging technique is best judged by evaluating x-z and y-z orthogonal sections through the dataset. We find the level of resolution, contrast, and detail preserved to be indistinguishable in the three dimensions. Therein lies the major strength of SIM: the in-plane tissue and cavity architecture preservation in SIM is comparable to that achieved in thin paraffin sections or confocal optical sections and its through plane resolution is currently unrivaled.

Comparison with Other Imaging Techniques

Current imaging techniques are not well suited to imaging millimeter scale samples at micron scale resolutions. Traditional histology has an extensive history of specific stains, both fluorescent and colorimetric, but is quite laborious and requires the viewing and/or photographing of hundreds to thousands of physical sections to gain an appreciation of complex three-dimensional structures. Methods that capture images of these sections and computationally warp them into three-dimensional volumes are of considerable use, but have not reported resolution or isotropicity similar to SIM [5, 6]. Previous efforts have also imaged the blockface of samples after physical sectioning, to build three-dimensional reconstructions of samples [1-4, 7]. SIM is distinguished from other block face approaches in the resolution, the contrast, and the tissue detail preserved through imaging. SIM resolves cells, subcellular structures, and can be used to label specific subpopulations of cells within an embryo, in contrast to lower resolution alternative methods relying on the inherent contrast in the tissue [3, 4]. Finally, the range of possible sample applications for SIM is broader; we have successfully imaged samples as delicate as early gastrulae chick embryos and samples as rigid as non-decalcified trabecular bone.

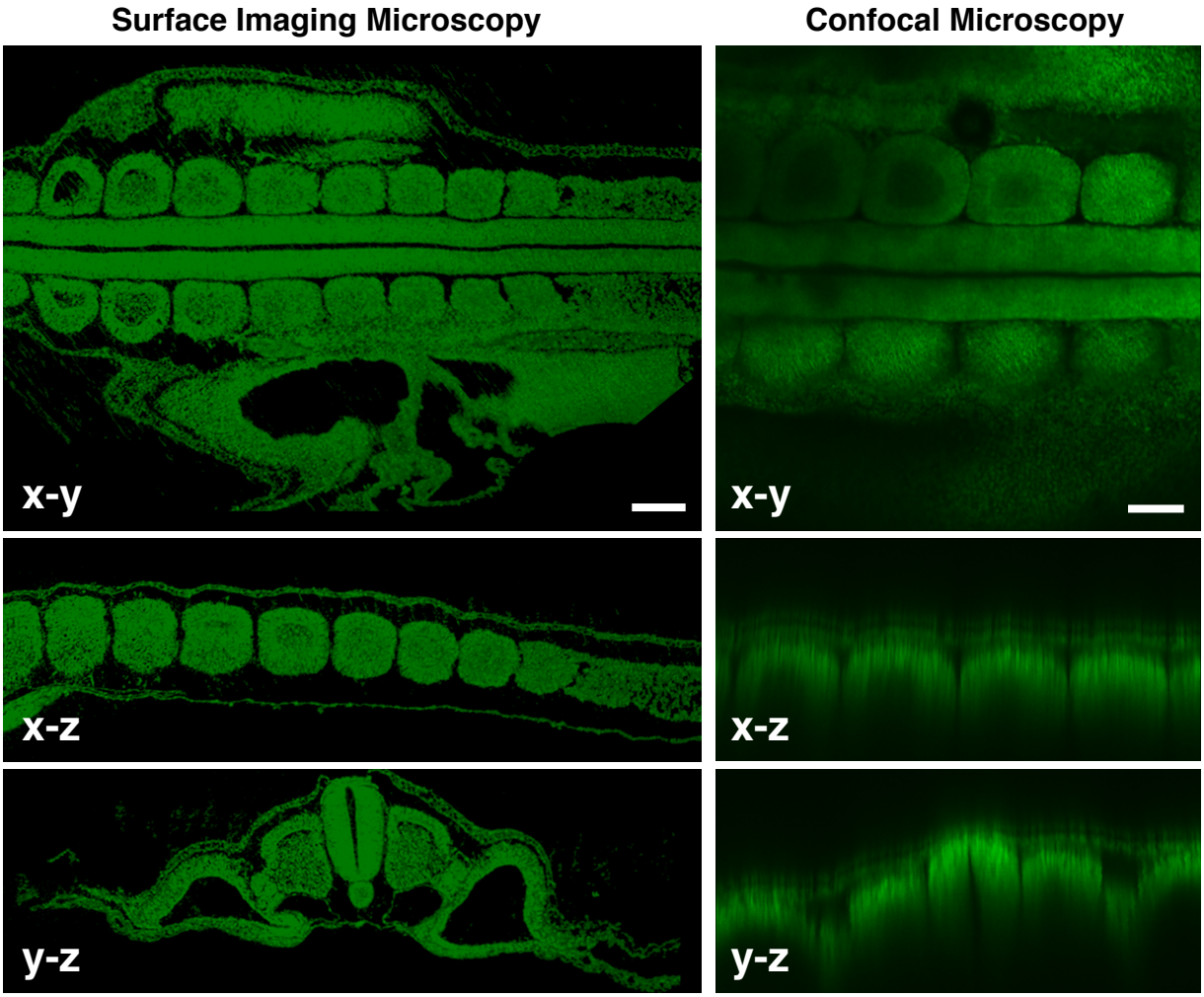
Several techniques exist which intrinsically collect three-dimensional volumes, rather than two-dimensional sections. Among them are optical coherence tomography (OCT), magnetic resonance imaging (MRI), and optical projection

tomography (OPT). OCT images backscattered light coming off a sample and has the potential to image 3 mm into a living specimen, but does so with 12-15 micron resolution and few options for specific contrast [8]. High-field MRI is excellent for imaging large specimens at 50-100 micron resolution. In fixed specimens, 35-50 micron resolution is achievable with long scan times and large magnetic fields [9]. OPT is an exciting new technique related to computed tomography that images fixed large samples that have been made optically transparent. It is unsuited to embryos containing very dense tissues such as cartilage or bone though, and has not been reported at cellular resolution [10]. It is currently a very exciting period in biological imaging, with rapid improvements being made across all imaging modes. SIM aids this progress by filling an unserved niche of cellular resolution on large samples, and should prove a highly complementary alternative to existing approaches.

Comparison of SIM with CLSM

To directly compare the performance of confocal laser scanning microscopy (CLSM) [11] with surface imaging microscopy, we imaged the same propidium iodide stained chick embryo with both techniques. The in-plane 2D images in Figure 5-4 (marked x-y) allow assessment of the image quality of the

Figure 5-4, Comparison of SIM with Confocal Microscopy: Comparative imaging of a chick trunk segment, stained with propidium iodide and imaged with both surface imaging microscopy and confocal laser scanning microscopy. Each technique is presented with three orthogonal views, starting with the original (x-y) plane of optical section, and following with computationally reconstructed views extracted from the resulting volume. Both datasets were collected with 1.7 micron optical resolution and sections were collected at 1.7 micron intervals. Note in particular the equivalence of the resolution, contrast, and level of detail in the x-y, x-z, and y-z views from the SIM dataset and the steep decline in image intensity as a function of depth into the dataset in the confocal dataset. Scale bar = 100 microns.



Bar = 100 microns

raw data in each imaging mode and show comparable image quality. The most striking differences are the shrinkage of structures in the SIM images, due to dehydration, and the larger overall field of view of the SIM images, which is due to the larger field of view of the surface imaging microscope compared to the Zeiss 410 CLSM. The shrinkage of tissue in alcohol is unavoidable and the shrinkage we observe is consistent with published norms [12].

The more informative views of the sample are the orthogonal x-z and y-z views through the resulting three-dimensional volume. The quality of the confocal images clearly falls off very quickly as a function of depth within the sample, even with the use of a water immersion C-Apochromat 10x objective lens. Even in regions of the volume where the quality is adequate, the through plane resolution is worse than the in-plane resolution. SIM, by contrast, has uniform image quality throughout the volume and has indistinguishable resolution and level of detail in the three directions. SIM in-plane image quality is not a function of depth or of the scattering, density, or degree of labeling of the tissue above or below the current plane of optical section. This has a trivial basis, in that there is no overlying tissue, but has profound consequences for image and dataset quality.

Practical Resolution of SIM Datasets

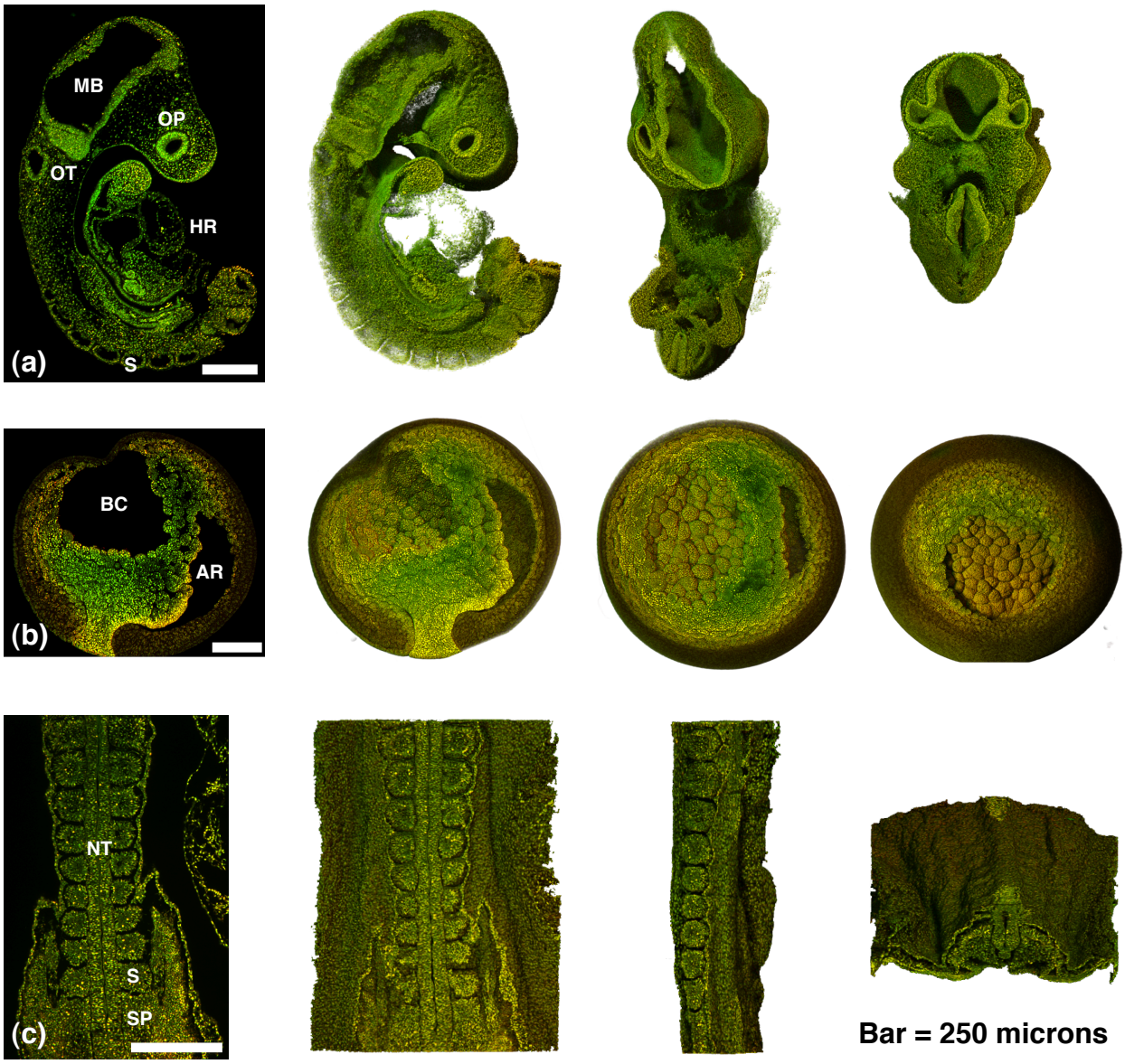
SIM can be performed on a variety of sample sizes and magnifications. These magnifications provide a useful resolution and field of view range that brackets

whole embryos at tissue resolution through small tissue segments at subcellular resolution. Low (2-4x) magnification allows ready identification of tissues and organs within small organisms and enables the study of their morphological development and relative orientation with respect to each other. Intermediate (10x) magnification enables the study or tissues at cellular or subcellular resolution. High (20-40x) magnification enables the study of small blocks of tissue at cellular or subcellular resolution.

Range of Systems Tested

Figure 5-5 depicts 2D raw sections and 3D reconstructions of an intact mouse and frog embryo and a segment of a chick embryo at 10x magnification. SIM routinely provides high-resolution, high-contrast images throughout embryos from each of these species at a wide range of stages. The cells of the mouse embryo in Figure 5-5a are clearly visible, and mesenchymal tissue is quite distinct from epithelial cell layers. This level of resolution provides a clear view of the relationship between developing tissues and organs in the context of an intact embryo. The gastrula stage frog embryo in Figure 5-5b is composed of relatively large cells, whose shapes and polarities are quite evident in three-dimensional reconstruction, especially those of the cells lining the blastocoel and archenteron. Additionally the germ layer organization of the frog is quite evident, in the

Figure 5-5, Volumetric Images of Embryos from 3 Model Systems: A broad range of sample types are readily imaged using surface imaging microscopy, including chick, mouse and frog embryos. All three embryos were stained with Resolution Standard Stain. **(a)** A two-dimensional raw image collected from a 9.5 days post coitum (dpc) mouse embryo and three orthogonal three-dimensional reconstructions of the resulting dataset, corresponding to sagittal, transverse, and frontal perspectives. Note the excellent preservation of lumenal structures in the midbrain (MB), the somites (S), the heart (HR), the optic vesicle (OP), and the otic vesicle (OT). **(b)** A two-dimensional raw image collected from a Stage 12 frog embryo and three-dimensional reconstructions of this late gastrula frog embryo, highlighting the blastocoel (BC) and the archenteron (AR). Note the reconstruction of the cell shapes on the floor of the blastocoel and in the lining of the archenteron. **(c)** A two-dimensional raw image and three orthogonal three-dimensional reconstructions of a trunk segment from a 16 somite stage chick embryo described in Figure 5-2. Note the clear developmental progression of somites (S), extending from the segmental plate (SP), adjacent to the neural tube (NT). Scale bar = 250 microns.


MB
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Bar = 250 microns

concentric epithelial shells surrounding the vegetal yolk mass. We have imaged chick embryos from Hamburger and Hamilton Stage 4-16, frogs from Stage 9-22, and mouse embryos from embryonic day 8.5-15.

Conclusions

We have demonstrated a new imaging technique based on the automated serial collection of thin optical sections from the freshly cut surface of a fluorescently labeled sample embedded in a highly opaque polymer. The realignment accuracy is sub-resolution and there is little or no detectable variation in image quality throughout the volume. The voxels of the dataset are highly isotropic, allowing digital resampling of the dataset from arbitrary virtual planes of section. This technique works across an order of magnitude in sample size and resolution, with improvements on track to significantly extend this range. SIM provides a unique combination of large sample size with high resolution and specific contrast.

We have demonstrated, in three different vertebrate model systems, that the practical resolution of a surface imaging microscope is sufficient to image small blocks of tissue with subcellular detail, large blocks of tissue with cellular resolution, and whole embryos with cellular to tissue resolution. We have also established that SIM produces high-contrast, high-resolution, high signal-to-noise ratio images with five different fluorescent dyes. Finally, direct comparisons, on the same block of tissue, demonstrate the advantages of SIM vs. confocal

microscopy, particularly in terms of depth of penetration and maximal sample size.

We expect that the combined ease of review of the primary data and ease of transfer of data should allow SIM to provide novel opportunities for collaborations in which phenotypes can be rapidly evaluated and extensively analyzed by experts at different institutions. The digital 3D models that result from SIM datasets can also be used as a structural scaffold to integrate other categories of information [13].

Future technical challenges within the reach of SIM include imaging green fluorescent protein (GFP) expression in intact embryos, imaging antibody revealed protein expression, and fluorescent in situ hybridization to mRNA probes. We have already achieved some success imaging the clonal distribution of fluorescent dextrans within frog embryos. Improvements in optical sensitivity and whole mount labeling techniques should enable these advances in the near term. We are also currently working on developing organic dye labeling approaches capable of revealing the plasma membranes of cells throughout the embryo. In combination with the nuclear dyes that have proven successful in this study, these should enable clear visualization of all of the cells in intact vertebrate embryos. Biological problems that we expect SIM to address to in the near future include somitogenesis, gastrulation, and organ morphogenesis. In each case complex morphogenetic rearrangements are taking place

throughout large regions of the embryo, and a cellular level of detail is required to understand the process.

Experimental Procedures:

Sample Preparation

White leghorn chicken eggs were incubated at 37° C for two to three days until they reached the desired stage of development, typically 16-25 somites. They were then harvested into ice-cold Howard Ringer's Solution, and fixed overnight in fresh 4% PFA at 4°C. Following fixation the embryos were washed three times for 30 minutes each in PBS. Embryos of Xenopus laevis were obtained, cultured, and dejellied according to standard techniques [14]. Staging was done according to the Niewkoop and Faber normal tables of Xenopus development [15]. Embryos were cultured to the desired stage, fixed in Bouin Fixative (75% picric acid, 25% formaldehyde (37-40%), 5% glacial acetic acid) overnight, and rinsed exhaustively in (50% ethanol, 50% water with 50mM NH4OH). The frog embryos were then dehydrated to absolute ethanol, stored overnight in a –20°C freezer, changed to fresh ethanol, and rehydrated to PBS. Embryos from B6D2F1 hybrid mice were obtained from timed matings where noon of the day when the copulatory plug was found is designated as 0.5 dpc. Embryos were harvested into ice cold PBS and transferred into fresh 4% paraformaldehyde for fixation overnight at 4°C. Embryos were rinsed in PBS, dehydrated to 100% ethanol, and rehydrated to PBS.

Sample Labeling

Embryos were fixed in 4% PFA, rinsed in PBS, stained in either propidium iodide (50 micrograms per mL for 1 hour), DAPI (10 micrograms per mL for 1 hour), Syto 62 (1:50 dilution for 1 hour), or Topro3 (1:50 dilution for 1 hour) (all Molecular Probes, Eugene, Oregon). Samples labeled with Resolution Standard Stain (Resolution Sciences Corporation, Corte Madera, CA) were stained for 6 hours. Dilutions were into PBS. Under all staining conditions, embryos were rinsed in PBS, then dehydrated to either methanol or ethanol.

Comparison to Confocal Microscopy

Chick embryos were fixed and stained in propidium iodide (50 micrograms per mL for 1 hour), then imaged in PBS on a Zeiss 410 confocal microscope, using a C-Apochromat 10x water immersion objective lens. Image acquisition was 512x512 pixels, with the pinhole at 1 Airy unit, in-plane resolution at 1.7 microns, and 1.7 micron intervals between optical sections. The embryos were then restained in propidium iodide and imaged by SIM with a 10x Plan Apochromat air objective lens, with 1.77 micron cubic isotropic voxels. Both image stacks were imported into the LSM 510 imaging software, version 3.0 (Carl Zeiss, Inc, Thornwood, New Jersey) and all comparisons were made therein.

Sectioning and Imaging

To prepare a sample for SIM imaging, it needs to be labeled, fixed, dehydrated, infiltrated, and embedded. We have imaged samples fixed in PFA (4%

paraformaldehyde in phosphate buffered saline (PBS), pH 7.4), formalin, Carnoy's Fixative, and Bouin Fixative [12]. Samples have been dehydrated to methanol, ethanol, or isopropanol. This flexibility in sample preparation enables customization to the specific needs of different tissue specimens, allowing for more optimal preservation of cellular detail and tissue integrity. Additionally, SIM is ideally suited to samples of varying density as it is able to section through dense and loose tissue with minimal chatter or unevenness.

Under all staining conditions, embryos were rinsed in PBS, then dehydrated to either methanol or ethanol. Following dehydration, embryonic tissues were equilibrated with a mixture of Resolution Standard Embedding Polymer and Resolution Standard Opacifier (Resolution Sciences Corporation, Corte Madera, CA) for 6 hours to allow for cellular infiltration. Opacifier is added to polymer to 82% of saturation to form 100% Stock Opacified Polymer (SOP), which may be stored in aliquots at -4 degrees Celsius for up to 3 months. Prior to embedding, the SOP is brought to room temperature, and then a working polymer is produced by adding to the 100% SOP sufficient additional fresh unopacified polymer to produce the final opacifier concentration appropriate to a given microscope objective. These concentrations have been empirically determined for a set of standard tissues; typical values for different objective lenses are 2x =10%, 4x=20%, 10x=30%, 20x=40%, 40x=50% opacity (Nikon Plan Apochromat series objective lenses). The embedding polymer was cured for 8 hours at 70° C.

The embedded tissue was mounted onto a vertically oriented translation stage assembly, which draws the sample over the edge of a diamond knife, removes the section with vacuum, then repositions the block face in its original location, in the field of view of a Nikon E600 fluorescence microscope with a Plan Apochromat 4x, 10x, or 20x objective lens. Images were collected to a Kodak Megaplus Model 4.2i charge coupled device (CCD) camera. Wavelength selection is accomplished with interference filters and dichroic beam splitters (Chroma Corporation, Brattleboro, VT). Individual images were reassembled into a three-dimensional volume that can be visualized and quantified on Resolution ResView 3.1 software. Alternatively, a series of two-dimensional images can be re-exported and computationally reconstructed into a three-dimensional volume that can be manipulated in other image processing programs. Because of the high inherent contrast between the sample and the block face, and the absence of a significant out-of-plane component in the image, there is no need for automated or manual deconvolution techniques to reconstruct accurate three-dimensional renderings from SIM datasets. The entire imaging process, from loading the block to finishing image collection, takes approximately three to six hours, depending on the number of sections that need to be cut.

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