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High Resolution 4D Imaging of Metanephric Embryonic Kidney Morphogenesis

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Abstract

We have developed a new method for imaging whole metanephric organ culture in 3D over time at cellular resolution. This method combines the use of the newly available generation of IR optimized long working distance high NA objectives and multiphoton fluorescence microscopy with a new method for vital staining of metanephric organ cultures with bodipy ceramide. This method allows all cells in the organ culture to be visualized over time, enabling detailed observation of tissue morphogenesis. This method offers a powerful new approach for visualizing and understanding early events in renal development and for extending observations made in genetically manipulated models.

Whole metanephric organ culture was developed in the 1950s (1) and has been the most important in vitro model system to date for studying kidney development. Whole metanephroi, isolated from embryonic mice at embryonic day 11.5 to 13.5 (E11.5 to E13.5), are cultured at an air-media interface. In response to signals from the metanephrogenic mesenchyme, the epithelial ureteric bud grows into the mesenchyme and branches repeatedly. Tips of the branching ducts induce a series of differentiation events in the surrounding mesenchyme, ultimately resulting in the formation of uriniferous tubules (2). These cultures have been essential in development of the current understanding of not only the morphological events, but also the genetic and molecular regulation of kidney development. Further, the expression of green fluorescent protein (GFP) transgenes and the advent of transgenic animal models has made analysis of changes in protein distribution over time (3) and analysis of developmental consequences of specific mutations possible using these organ cultures.

High resolution three-dimensional imaging of fixed embryonic kidney tissues has advanced considerably in the past decade. The application of multiphoton microscopy provided the ability to image more deeply into samples with reduced photobleaching (4) and this approach was made even more powerful when combined with advanced visualization techniques (5) for exploration of complex structures within this tissue. Recently, application of optical clearing to imaging of fixed kidney tissues increased imaging depth even further with greatly improved image quality at depth (6).

These high resolution approaches have not been possible to apply to live imaging of embryonic kidney cultures. When in culture, the embryonic kidney grows at an air-media interface on a filter, drawing nutrients from a pool of medium below. The typical approach has been to image these cultures using low magnification, low numerical aperature (NA) air immersion objectives (7-10). This approach has produced significant advances in understanding of nephrogenesis, but because resolution in these studies is limited by the low NA of air objectives, they do not reveal detail at the level of single cells. Here we demonstrate a new method that accomplishes high resolution imaging of developing embryonic kidney cultures in 3D over time.

To visualize morphogenesis in the metanephric embryonic kidney organ culture, we used BODIPY® FL C5-ceramide (bodipy ceramide), a vital fluorescent label commonly used in developmental studies of zebrafish, C. Elegans, and chick (11-13). When used to label cultured cells, dye accumulates in the plasma membrane and in the Golgi complex. When used to label whole embryos, dye also freely diffuses in the interstitial spaces between cells, allowing all cells of an embryo to be visualized and bulk tissue morphogenesis across the organism at the single

cell level to be observed. To our knowledge this method has not previously been applied to visualization of metanephric embryonic kidney culture morphogenesis.

We isolated kidneys from E10.5 to E11.5 mouse embryos (14). Immediately after dissection, kidneys were placed in 0.5 ml. of Hepes buffered medium [DMEM/Ham's F-12 1:1, with 10% FBS, 2 mM L-glutamine, 1uM dexamethasone, and PenStrep] (Sigma, D-6421) containing 100 μM bodipy ceramide [(N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine (BODIPY® FL C5-ceramide)] (Invitrogen, D-3521) and incubated in a 12-well dish on ice for 4 hours. This medium contains HEPES and sodium bicarbonate, can be used with or without CO_2, and is used for isolation, culture and imaging. Kidneys were then placed on clear transwell filters with 0.4 μm pore size and 12 mm diameter (Corning snapwell inserts Cat.No. 3801). The reservoir under the filter was filled with 25 μM bodipy ceramide in medium and kidneys were incubated at 37^{O}C with 5% CO_2 until imaging 4-24 hours later. Minimum incubation after placement on filters was 4 hours to allow attachment to the filter. Four labeled kidneys were visually compared with unlabeled sibling kidneys in three separate experiments. No morphological differences in growth and development were seen. While some subtle effects no doubt occur and these potential effects merit future consideration, we posit that this label has the potential to be as useful in the study of embryonic kidney development as it has been in other embryonic developmental studies. Bodipy ceramide labeled embryonic kidneys were used to develop our technique for high resolution 4D imaging of metanephric embryonic kidney cultures.

Metanephric embryonic kidney cultures present a unique challenge for high resolution live imaging. Yet, the basic issues that must be addressed are the same as those that must be addressed for successful imaging of any living cell, embryo or organ culture. First, physiological

conditions for the culture must be maintained on the microscope stage. Second, the correct equipment must be chosen for the specific demands of the imaging. Third, the equipment and culture conditions must remain stable over an extended period of time. To address these issues, we developed the method illustrated in Figure 1. The stage was shielded from drafts and a stage heater or stage-top incubator and an objective heater, both set to 37^{O}C, were allowed to thermally equilibrate for at least one hour. Immediately prior to imaging, the filter insert containing the labeled embryonic kidney was snapped free of its supports (Corning snapwell inserts Cat.No. 3801) and the detached filter insert was placed over a small pool of fresh medium in a glass bottom dish (MatTek P35G-1.5-14-C or P35-0.17-14C). Using this specific insert and dish, the insert fits slightly down into the well of the glass bottom dish, with a small space remaining for medium. To maintain humidity and prevent evaporation of the limited pool of medium, the dish was sealed with parafilm (Figure 1).

Imaging was performed from below using an inverted microscope. This approach has only recently been made possible by the availability of a new generation of IR optimized long working distance high NA water immersion objectives coupled with multiphoton microscopy. The long working distance is required to reach the sample in this configuration and multiphoton illumination is needed to image deeply into the highly scattering embryonic kidney tissue. The Olympus 25x, NA 1.05 IR optimized water immersion objective with a 2 mm working distance and an excitation wavelength of 900 to 950 nm were used.

This imaging method was tested on two systems, an Olympus FV1000 MPE system equipped with a MaiTai BB DeepSee laser (Spectra-Physics, Irvine CA) (Figure 2 A-C) and an Olympus FV1000 confocal system, custom modified for multiphoton imaging at the Indiana Center for Biological Microscopy, equipped with a MaiTai BB laser (Spectra-Physics) and

highly sensitive GaAsP photo multiplier tubes (Hamamatsu, Hamamatsu City, Japan) (Figure 2 D-F). Similar results were obtained on both systems. To avoid evaporation of the water droplet, immersion oil with the refractive index of water (Series AAA Refractive Index Matching Liquid nd 1.330 at 25 deg C, Cargille Laboratories, Cedar Grove, N.J.) was used.

To minimize phototoxicity, imaging was performed with no averaging. While focused on an optical section in the middle of the embryonic kidney, laser power was adjusted to utilize the full dynamic range of the detector with minimal saturation. The correction collar on the objective was then adjusted to maximize brightness while focused in the deepest portions of the embryonic kidney (15). The measured axial resolution of the objective was 1.8 μm so initially we obtained optical sections at 1μm intervals, near the sampling rate needed to satisfy Nyquist. Unfortunately, at this sampling rate of up to 100 images per time point, development of labeled embryonic kidneys slowed after having been imaged four to six times. By obtaining optical sections at 2 μm intervals, 12 to 16 time points were acquired without inhibiting development (Figure 2 D-E). Number of time points collected was ultimately limited by loss of fluorescence due to photobleaching. Also tested was vital Hoechsts labeling (Figure 2 C).

The method we have developed allows analysis of morphogenetic cell movements of the entire whole metanephric organ culture in 3D over time at cellular resolution. This has been made possible by use of the latest generation of long working distance, high numerical aperature, IR optimized objectives coupled with multiphoton microscopy. With the ability to image all cells within the developing embryonic kidney over time, it is now possible to perform optical flow analysis and single cell tracking in 3D over time, enabling researchers to definitively determine how cell dynamics during branching morphogenesis result in formation of uriniferous tubules and glomeruli. Particularly exiting is the potential of this method when applied to high resolution

imaging of GFP-transgenics (3) using bodipy ceramide as counterstain, as has been done in zebrafish (16).

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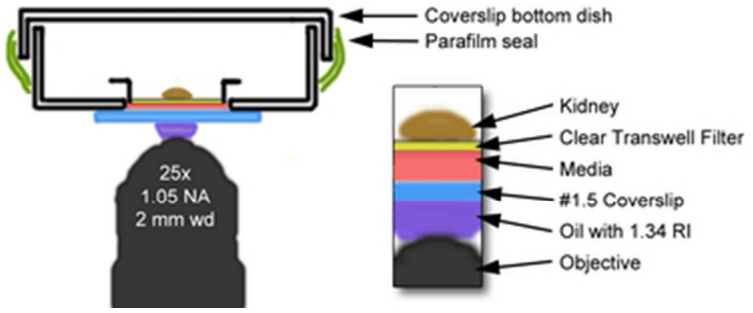
Disclosure

The authors declare no competing interests.

Figure Legends

Figure 1. Diagram of system for live imaging of embryonic kidney at high resolution. A 25x water immersion objective with numerical aperature of 1.05 and working distance of 2 mm, optimized to pass IR excitation wavelengths, is mounted on an inverted Olympus FV1000 microscope equipped for multiphoton imaging. Oil with the refractive index of water optically couples the objective to the coverslip bottom dish. Coverglass thickness is 170 μm. Reservoir of medium above glass is 1000 μm in thickness. The clear transwell filter thickness is 10 μm. The embryonic kidney maximum thickness is about 100 μm. Total thickness of embryonic kidney, filter, media and coverslip is 1280 μm, which is within the working distance of the objective. Stage, stage heater and objective heater are not illustrated.

Figure 2. Live embryonic kidneys imaged at high resolution in 3D over time. Bodipy ceramide labeled (A,B and D-F) and Hoechsts labeled (C) live embryonic kidneys imaged beginning at day 0 (A,B), day 1 (D-F), and day 2 (C) in culture. Embryonic kidneys were imaged at intervals of 30 min (A,B) or one hour (D-F). Time after initiation of imaging is shown. Bodipy ceramide labeled embryonic kidneys could be imaged in 3D over time (A,B and D-F). Hoechsts labeled embryonic kidneys (C) could only be imaged one to two time points before fragmentation of nuclei was observed. All images are single planes from near the midpoint of the volume. Bars = 100 μm.



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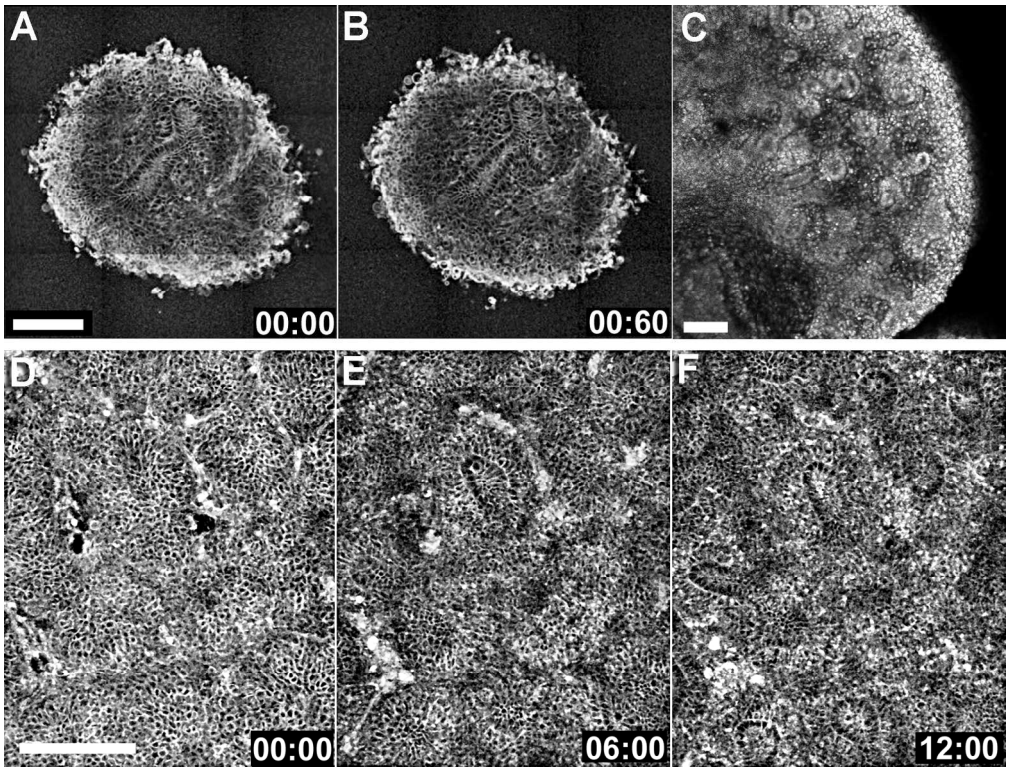

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177x135mm (300 x 300 DPI)