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High resolution 4-dimension imaging of metanephric embryonic kidney morphogenesis

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High-resolution three-dimensional imaging of fixed embryonic kidney tissues has advanced considerably in the past decade. Here we developed a new process for imaging whole metanephric organ culture at cell resolution in three dimensions over time. This technique combines the use of the newly available generation of infrared-optimized long working distance, high numerical aperture objectives and multiphoton fluorescence microscopy with a new system for vital staining of metanephric organ cultures with bodipy ceramide. This allows all cells in the organ culture to be visualized over time, enabling detailed observation of tissue morphogenesis. Thus, our method offers a powerful new approach for visualizing and understanding early events in renal development and for extending observations made in genetically manipulated models.

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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 the signals from the metanephrogenic mesenchyme, the epithelial ureteric bud grows into the mesenchyme and branches repeatedly. The 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 the 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 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 aperture air-immersion objectives.[7–10] This approach has produced significant advances in the understanding of nephrogenesis, but because resolution in these studies is limited by the low numerical aperture of air objectives they do not reveal the details at the level of single cells. Here we demonstrate a new method that accomplishes high-resolution imaging of

developing embryonic kidney cultures in three dimensions 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 the developmental studies of zebrafish, *C. elegans*, and chick.[11–13] When used to label cultured cells, the dye accumulates in the plasma membrane and in the Golgi complex. When used to label whole embryos, the 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 the visualization of metanephric embryonic kidney culture morphogenesis.

We isolated kidneys from E10.5 to E11.5 mouse embryos.[14] Immediately after dissection, the kidneys were placed in 0.5 ml of Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered medium (DMEM/Ham's F-12 1:1, with 10% FBS, 2-mmol L-glutamine, 1-μmol dexamethasone, and PenStrep) (Sigma-Aldrich, St Louis, MO, D-6421) containing 100-μmol bodipy ceramide ((N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl) sphingosine (BODIPY FL C5-ceramide)) (Life Technologies, Invitrogen, Grand Island, NY, D-3521) and incubated in a 12-well dish on ice for 4 h. This medium contains Hepes and sodium bicarbonate, can be used with or without CO_2, and is used for isolation, culture, and imaging. The kidneys were then placed on clear transwell filters with 0.4-μm pore size and 12-mm diameter (Corning, Corning, NY, snapwell inserts Cat.No. 3801). The reservoir under the filter was filled with 25-μmol bodipy ceramide in medium and the kidneys were incubated at 37 °C with 5% CO_2 until imaging 4–24 h later. The minimum incubation period after placement on the filters was 4 h 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. Although 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 °C, were allowed to thermally equilibrate for at least 1 h. Immediately before 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 P35-0.17-14-C). 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 the medium. To maintain humidity and prevent evaporation of the limited pool of medium, the dish was sealed with parafilm (Figure 1).

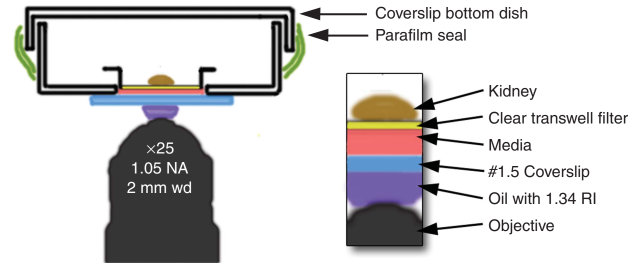

Figure 1 | Diagram of system for live imaging of embryonic kidney at high resolution. A ×25 water immersion objective with numerical aperture of 1.05 and working distance of 2 mm, optimized to pass infrared excitation wavelengths, is mounted on an inverted Olympus FV1000 microscope equipped for multiphoton imaging. Oil with the refractive index of water optically couples the objective to the coverslip bottom dish. Coverglass thickness is 170 μm. The reservoir of the medium above the glass is 1000 μm in thickness. The clear transwell filter thickness is 10 μm. The embryonic kidney maximum thickness is about 100 μm. The total thickness of the 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.

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 infrared (IR)-optimized long working distance, high numerical aperture 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 ×25, numerical aperture 1.05 IR-optimized water immersion objective with a 2-mm working distance and an excitation wavelength of 900–950 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) (Figures 2a–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) (Figures 2d–f). Similar results were obtained on both systems. To avoid evaporation of the water droplet, immersion oil with the

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