

Novel fixed z-direction (FiZD) kidney primordia and an organoid culture system for time-lapse confocal imaging

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ABSTRACT

Tissue, organ and organoid cultures provide suitable models for developmental studies, but our understanding of how the organs are assembled at the single-cell level still remains unclear. We describe here a novel fixed z-direction (FiZD) culture setup that permits high-resolution confocal imaging of organoids and embryonic tissues. In a FiZD culture a permeable membrane compresses the tissues onto a glass coverslip and the spacers adjust the thickness, enabling the tissue to grow for up to 12 days. Thus, the kidney rudiment and the organoids can adjust to the limited z-directional space and yet advance the process of kidney morphogenesis, enabling long-term time-lapse and high-resolution confocal imaging. As the data quality achieved was sufficient for computer-assisted cell segmentation and analysis, the method can be used for studying morphogenesis *ex vivo* at the level of the single constituent cells of a complex mammalian organogenesis model system.

KEY WORDS: Kidney, Organ culture, Time-lapse, Organoid, Imaging

INTRODUCTION

High-resolution confocal imaging is an important means of studying the cellular behavioural dynamics of organ morphogenesis, a process that involves division, migration, death and changes in shape of the cells. Yet, we have a poor understanding of how these cellular events establish the shapes of the eventual organs. The rapid development of microscopic imaging (Huisken et al., 2004) and automated image analysis technologies (Eliceiri et al., 2012) has provided the basis for achieving a better understanding of these processes, as has been shown in certain model organisms (Khan et al., 2014).

In the traditionally used Trowell setup, the explant is placed on a filter supported by a metal grid (Auerbach and Grobstein, 1958; Grobstein, 1955; Kispert et al., 1998; reviewed in Rak-Raszewska et al., 2015; Saxén, 1987), and more recently, commercial inserts have also been used for this purpose (Costantini et al., 2011). For high-resolution time-lapse imaging, however, more sophisticated ways of organizing the organotypic culture experiments using an on-stage microscope incubator are required. The branching morphogenesis and patterning of the ureteric bud (UB) has been monitored using Transwell inserts (Watanabe and Costantini 2004) and the low-volume method (Lindström et al., 2014), and there have been reports on UB cell behaviour (Packard et al., 2013) and the movement of progenitor cells during UB branching morphogenesis (Riccio et al., 2016) based on the use of Transwell inserts. The same system was used to investigate the effect of β -catenin levels during nephron patterning (Lindström et al., 2015) and for tracking the movements of cap mesenchymal cells (Combes et al., 2016). In all of these applications, however, the imaging of individual cells was limited, hindering the full use of these elegant models.

We report here a novel technique for culturing embryonic kidneys and organoids in a fixed z-direction culture (FiZD) set up in a restricted space between a Transwell filter and a glass coverslip. This technology provides high-quality single-cell-resolution time-lapse imaging and permits computer-assisted analysis of the assembly of complex cellular structures.

RESULTS AND DISCUSSION

The main disadvantage of traditional culture methods for conducting time-lapse imaging is poor light microscopy image quality. The reasons for this are the long focal length, the thickness of the tissue, and the air-liquid interface or Transwell membrane between the specimen and the objective (Fig. 1A).

This led us to consider whether restricting the ability of the sample to grow in the z-direction by use of a porous membrane (Fig. 1B) would enable better imaging quality. This proved to be optimal for supporting organogenesis and providing a high imaging quality (Fig. 2).

The z-direction was regulated by polyester beads that serve as spacers (Fig. 1B). We found empirically that a spacer of <20 μ m diameter did not prevent mechanical destruction of the samples (Fig. S2A–F) and 40 μ m beads gave greater variation in sample viability (Fig. S2G–L), whereas 70 μ m beads provided the best conditions for both high-quality imaging and optimal culture conditions (Fig. S2M–R). These observations indicate that the diameter of the beads must be optimized for each experiment according to the size and properties of the sample.

The formation of the renal vesicle, comma-shaped and S-shape bodies, and eventually segmented nephrons with Bowman's capsule and loops of Henle were also observed in intact kidneys and organoids in FiZD culture (Fig. 2A–H; Movies 1–3, 6–10).

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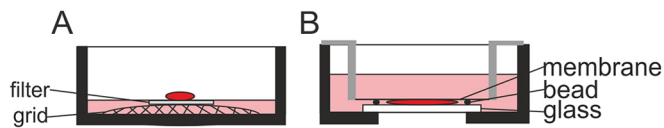


Fig. 1. Fixed z-direction system. (A) In the Trowell system the explant is placed on a filter supported by a grid holding the explant at the air-liquid interface. (B) In FiZD culture the explant is between a glass surface and a Transwell insert. Spacer beads are used to adjust the thickness of the tissue.

We addressed the question of how renal vasculature structures labelled with GFP reporter emerge in the FiZD setup of intact kidney (Movie 1), which was in line with previously published data (Halt et al., 2016).

We employed mT/mG;HoxB7Cre transgenic mice to study the degree of UB development, as highlighted by the *HoxB7Cre*-activated GFP expression in the epithelium of the UB (Movie 2).

FiZD was also used for studying nephron development in mT/mG;Wnt4Cre organoids. The metanephric mesenchyme was induced to undergo nephrogenesis by transient exposure to a GSK-3 α/β inhibitor. In such a setting the behaviour of individual GFP+ cells could be monitored in the images captured, allowing the analysis of cell movements and nephron patterning (Movie 3).

We next explored whether the data would allow computer-assisted cell segmentation. Confocal microscopic micrographs of GFP+ UB cells before (Fig. 3A) and after deconvolution (Fig. 3B) to improve the image resolution enabled cell segmentation (Fig. 3C). The GFP allowed tracking of the UB cell membranes as a result of the *HoxB7Cre*-mediated GFP activation (Movie 4).

The computer-assisted image analysis enabled several key morphogenetic parameters to be examined simultaneously (Fig. 4A,B). The data derived from the FiZD served to identify the speed and direction of UB cell migration as presented in a wind rose plot (Movie 5), as used in certain systems (Stegmaier et al., 2016). By analysing segmentation data it is possible to study cellular behavioural dynamics in detail during morphogenesis, including the processes that take place during the development of a nephron.

The low-volume method provided better quality images than Trowell culture (Sebinger et al., 2010) and was compared with the FiZD method. The FiZD and low-volume experiments were organized in one 6-well plate, with confocal imaging of intact kidney cultures and organoids (Fig. S3, Movies 6,7,10). To provide an objective comparison, the culture medium was changed daily in both types of culture, even though this is not required for the FiZD system. The results demonstrate the benefits of the FiZD system: better overall imaging quality, easy medium change without affecting the sample, reduced thickness of the sample and perfect stability of the image. Additionally, several samples could be assembled simultaneously in one FiZD setup with precise control of their position, whereas the low-volume method does not allow more than one sample per silicon chamber (note the fusion of what were initially two organoids in Movie 10, right panel). The FiZD method was also compared with the Transwell culture method (Movie 11). However, due to the low signal the laser power had to be doubled and this was toxic to the kidney rudiment (Fig. S4). To present the FiZD time-lapse data at the highest possible resolution, we made a short time-lapse of the developing nephrons (Movie 8), 3D structural analysis of the same developing nephron was provided by displaying all the z-layers at one point in time (Movie 9).

The capacity of the kidney to develop under FiZD culture conditions could be attributed to the conditions under which this occurs during normal nephrogenesis, when the nephron takes shape

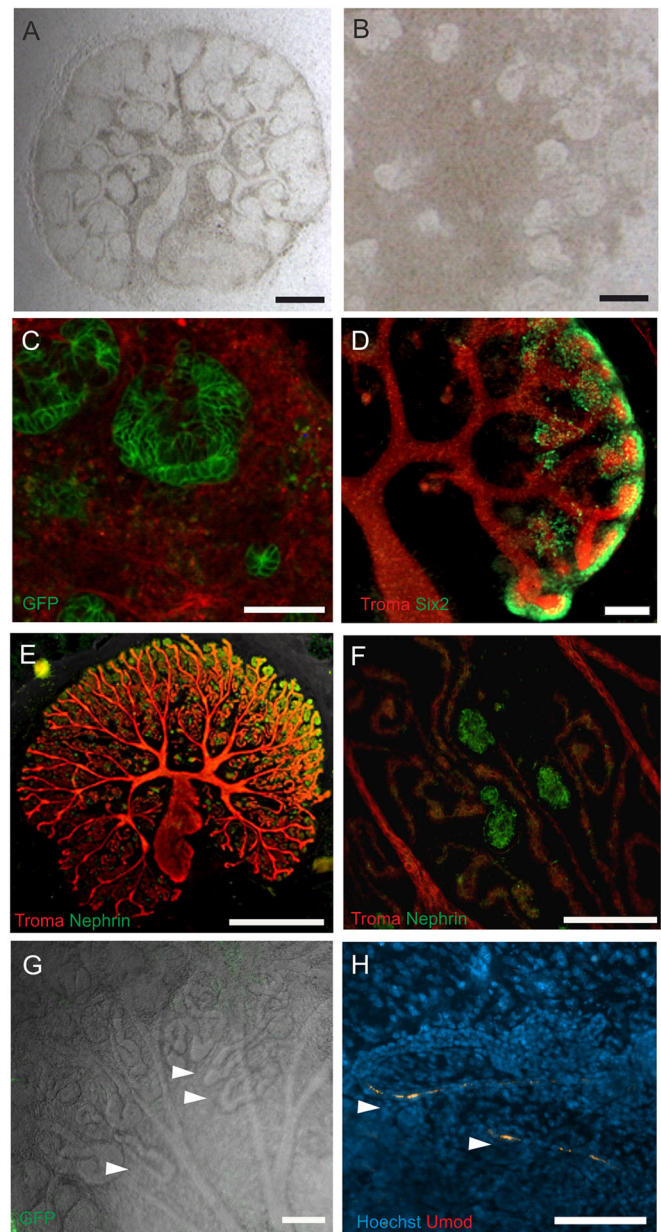


Fig. 2. Kidney morphogenesis progresses well in FiZD culture. Embryonic kidneys (A,D-G) and organoids (B,C,H) were grown in FiZD culture. (A) Brightfield micrograph of an intact embryonic kidney, and (B) kidney organoid cultured for 7 days. (C) Snapshot of the time-lapse image stack depicting *Wnt4Cre*-activated GFP expression in the assembling nephrons on the fourth day of FiZD culture. (D) Six2 and Troma-1 (Krt8) staining highlight nephron precursors and UB bifurcations, respectively, in a 7-day FiZD culture. (E) Kidney rudiment FiZD-cultured for 12 days. Troma-1 and Nephlin staining depict the UB bifurcations and podocytes, respectively. (F) High-power magnification of the Nephlin+ podocytes and Troma-1+ UB in E. (G) Frame from the time-lapse image stack of *Flk-GFP* endothelial cells. Henle's loop-like structures (arrowheads). (H) Umod+ loops of Henle (arrowheads) in an organoid cultured for 7 days. Umod and Hoechst stainings. Scale bars: 100 μ m in A-D,F-H; 1000 μ m in E.

during its assembly. It has been shown that the viscoelasticity of the tissue, and thus also its rigidity, depends on the composition and crosslinking of the extracellular matrix components and their binding to cells (Forgacs et al., 1998; Phillips and Steinberg, 1978).

As judged by several organogenesis indicators presented here, kidney morphogenesis advances well under FiZD conditions, and at

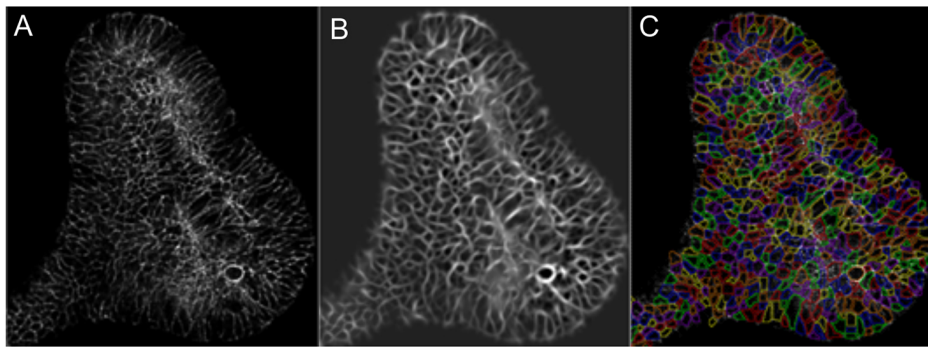


Fig. 3. FiZD culture-generated image data stacks can be subjected to computer-assisted cell segmentation. Time-lapse images were captured at 5 min intervals and processed with a program developed in Matlab. (A) Frame of the 3D image stack. (B) Ridge-enhanced image after deconvolution. (C) Cell boundaries highlighted by the cell segmentation program.

the same time the FiZD setup provides a superior capacity for image quality due to the reduction in tissue thickness. Here, multiple tissue samples can be cultured simultaneously and organs can be fixed in defined positions prior to culture. The culture medium can be changed or supplemented with given factors in a manner that does not disturb the development of the tissue or its capacity to retain its initial position. Moreover, the relatively large volume of the

wells and lower laser power enables long-term imaging. Most importantly, the FiZD is well suited for microscope stage incubation system culture and provides an excellent platform for high-resolution confocal imaging, automatic cell segmentation, tracking and image quantification that would also be suitable for use with other organotypic cultures (Prunskaitė-Hyyryläinen et al., 2016) and organoids.

MATERIALS AND METHODS

Mouse models and dissection of kidneys

The embryonic kidneys were dissected from wild-type CD-1 embryos or crosses between *Tie1Cre* (Gustafsson et al., 2001), *Hoxb7Cre* (Yu et al., 2002), *Wnt4Cre* (Shan et al., 2010), *Flkl-GFP* (Licht et al., 2004), *GFP* (Hadjantonakis et al., 1998) or *tomato floxed Rosa26 Green fluorescent protein (GFP) (mT/mG)* reporter mice (Muzumdar et al., 2007), as described by Junttila et al. (2015). Animal care and procedures were in accordance with Finnish national legislation for the use of laboratory animals, the European convention for the protection of vertebrate animals (ETS 123) and EU Directive 86/609/EEC.

Embryonic kidney organoids

An organoid can be defined as an *in vitro* 3D aggregate derived from primary tissue (Auerbach and Grobstein, 1958; Junttila et al., 2015; Unbekandt and Davies, 2010; Vainio et al., 1992), embryonic stem cells (Eiraku et al., 2008) or induced pluripotent stem cells (iPSCs) (Morizane et al., 2015; Takasato et al., 2015), which are capable of self-renewal and self-organization, and exhibit similar organ functionalities as the tissue of origin (Fatehullah et al., 2016). In our experiments we used organoids derived from primary cell cultures isolated from embryonic metanephric mesenchyme, as described by Junttila et al. (2015), except that 10 μ M BIO (6-bromoindirubin-3'-oxime, Sigma) was used for induction.

Embryonic kidney organoids derived from frozen primary cells

The organoids in the experiments presented in Fig. 2H and Movie 10 were made using frozen primary cells. Dissociated embryonic day (E)11.5 mesenchyme or intact E13.5 mT/mG kidneys were suspended in 20% DMSO/80% FBS and frozen in cell-freezing containers at -80°C and transferred the next day to liquid nitrogen. Upon usage the cell vial was quickly warmed, the cells washed twice with medium and then pelleted (4 min at 1380 g) to make organoids, as in the usual protocol. The intact kidneys were dissociated during the freezing/thawing process. When whole kidneys were used the cell suspension included UB cells, which induced the nephrogenesis.

Assembly of the tissue culture

The FiZD conditions were set up in 6-well CellStar plates (Greiner Bio-One) (Fig. S1). A hole (20 mm diameter) was drilled at the bottom of wells and glass coverslips (24 \times 24 mm) were glued to the upper side of the bottom with either dental wax or Histoacryl glue (Braun, 1050052). The coverslips were cleaned with ethanol and sonication to promote affixation of the organ rudiments to the coverslip glass (supplementary Materials and Methods). The culture plates were rinsed with ethanol, distilled water and dried in a UV hood.

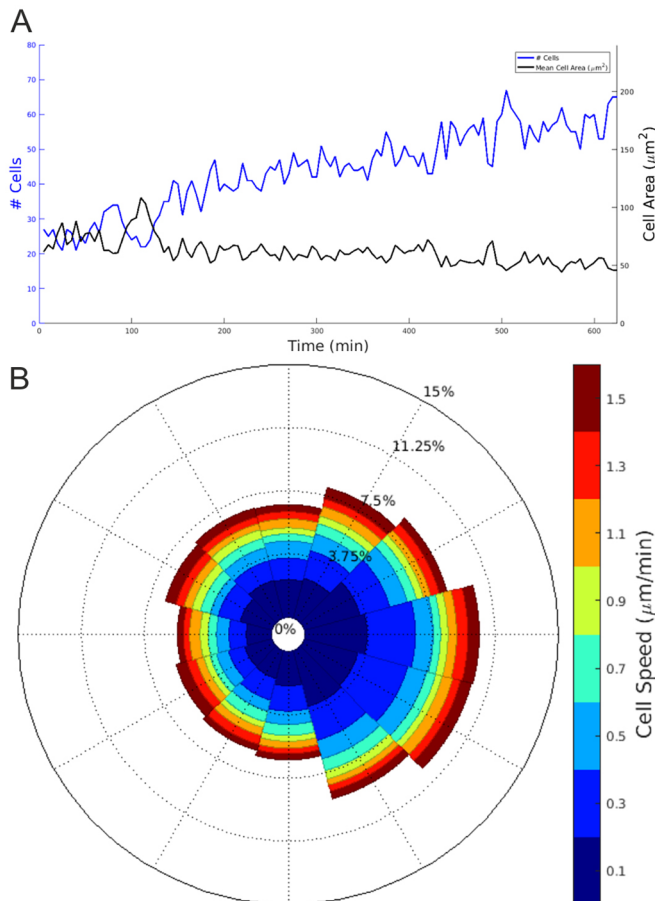


Fig. 4. Segmented FiZD culture-based image data enable analysis of kidney morphogenetic parameters. (A) Increase in the number of UB tip cells in the right-hand side of the growing kidney (blue), and the mean cell area (black). (B) Wind rose plot illustrating the direction of UB tip cell migration. The lengths of the spokes of the wind rose plot indicate the proportions of cells moving in a given direction and the thicknesses of the colour bands within a spoke indicate their speed distribution. Both wind rose and cell count plots show that the kidney is growing towards the right, as the number of cells in the right half is increasing and more cells are moving towards the right. The data correspond to the single z-projection presented in Movie 3.

Kidney organoids incubated overnight in a microfuge tube or E11.5 to E13.5 embryonic kidneys were arranged on the lower side of the Transwell insert membrane (Corning, 3450) and the excess volume of PBS was aspirated. Polystyrene beads (Corpuscular, 100263-10) were mixed with Matrigel on ice and a few microliters were added to the samples with care not to disturb their position on the filter. Matrigel can aid fixation of the specimen in a defined position, but it is not required (see Fig. 2B,D with Matrigel, and Fig. 2H without) and it did not influence morphogenesis. Next, the insert was turned so that the samples were face down, positioned in a well, and pressed gently into place. The rim of the insert was melted with a heated glass capillary at three points to fix the insert to the plate (Fig. S1). The well was filled with 2 ml DMEM (Gibco, 41965-039), 10% foetal calf serum (Gibco, 10500-064) and 1% penicillin/streptomycin (Sigma, P4333) and the plate was kept at 37°C and 5% CO₂. The culture setups were repeated a minimum of five times for each type of experiment. For the low-volume culture system we followed the method described in Sebinger et al. (2010), repeating the experiment 15 times. For imaging of the kidney cultures on the top of the filter (Costantini et al., 2011) the standard 6-well glass-bottomed plates were used (BD Falcon).

Time-lapse image capture

The culture plates were inserted into an on-stage incubator on a Zeiss LSM780 confocal microscope at 37°C and 5% CO₂. The time-lapse images were captured at 5–20 min intervals and processed with ZEN 2012 (blue edition) (Zeiss), Huygens Professional (Scientific Volume Imaging) and Fiji (Schindelin et al., 2012).

Whole mount immunostaining

The explants were transferred to 4% PFA for 20 min, washed in PBS, and stored at 4°C. For immunostaining, the samples were blocked for 1 h in 0.1% Triton-X100/1% BSA/10% goat serum/0.02 M glycine PBS at room temperature. Primary antibodies against Krt8 (1:100; clone Troma-I, AB_531826, Developmental Studies Hybridoma Bank), Nephlin (also known as Nphs1) (1:1000; a gift from Prof. Karl Tryggvason, Duke-NUS Medical School, Singapore), Six2 (1:200; 11560, Proteintech), Umod (1:20; LS-C150268, LSBio) and Pax2 (1:100; PRB-276P, Covance) were used for immunostaining.

After an overnight incubation at 4°C the samples were washed six times for 30 min in PBS and goat anti-rat Alexa Fluor 546 (1:1000; A11081, Molecular Probes) and goat anti-rabbit Alexa Fluor 488 (1:1000; A11008, Molecular Probes) or donkey anti-sheep NorthernLights NL557 (1:200; NL010, R&D Systems) were incubated 1 h at room temperature and washed several times with PBS. A Zeiss LSM780 microscope and Zeiss AxioLab were used for analysis and image capture.

Automatic image analysis

Embryonic UBs labelled with *HoxB7Cre*-activated GFP, highlighting the cell boundaries, were segmented and tracked using a program developed in Matlab (MathWorks). Hessian ridge enhancement (Hodneland et al., 2009) was used to enhance the cell membrane intensity, fill the membrane gaps and remove cytoplasmic fluorescence. H-minima transformation (Soille, 1999) was used to filter out local minima and the Watershed transformation (Meyer, 1994) for cell segmentation. The centroids of the segmented cells in the first frame were used to initialize the cell tracks and the Hungarian algorithm (Munkres, 1957) was used to associate detections with tracks. For details on segmentation of image data and preparation of wind rose plots, see the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

I.S. conceived and designed the project and experimental strategies. U.S. and I.S. performed the cultures, the time-lapse analysis, their processing and the biomarker analyses and were responsible for writing the paper. S.U.A. and J.H. performed the image analysis and A.D. and I.S. compiled the *HoxB7Cre* data sets. S.C. generated *Hoxb7Cre* mouse embryos A.R.-R. assisted in writing the manuscript. J.S. developed the *Wnt4Cre* mouse line, V.-P.R. assisted in the image analysis, and S.J.V. led the project, provided the infrastructure and finalized the writing of the paper.

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Supplementary information

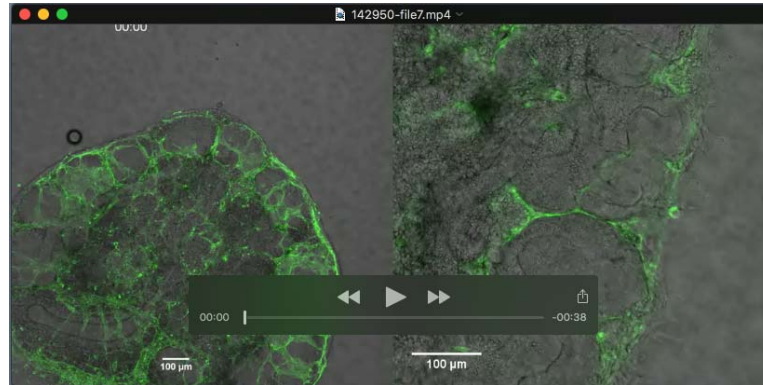
Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.142950.supplemental>

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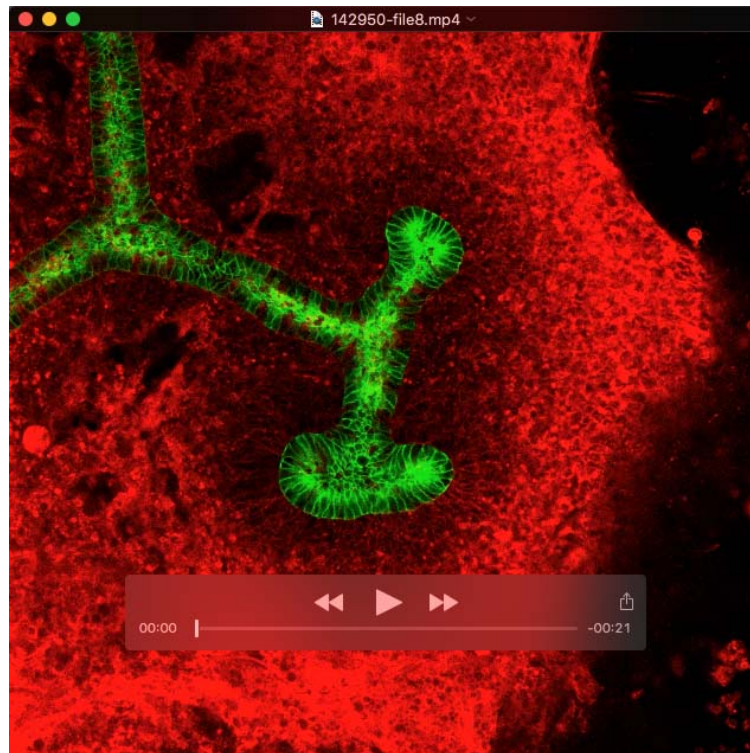
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Supplementary data



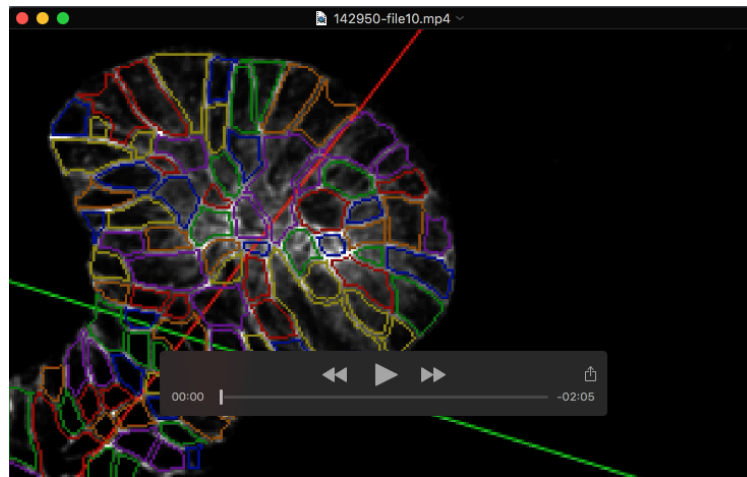
Movie 1. The renal endothelial cells survive and can be observed with high resolution in the FiZD culture. The embryonic kidneys from E11.5 *mTmG*; *Tie1Cre* embryos were dissected and cultured in the FiZD for 6 days. The movie presents the *Tie1Cre* induced GFP expression in the endothelial cells. A stack of 20 z-layers was captured using a 10x/0.45 Zeiss Plan-Apochromat objective, images acquired every 15min. In the movie one bright field focal plane and 5 GFP z-layers are merged. The panel on the right presents a zoomed in to actual resolution close up of a developing kidney where the endothelial cells migrating into vascular cleft of S-shape stage nephrons can be observed. Here one bright field plane and one GFP z-layer are merged. Note that also some blood cells are expressing GFP signal and some of the highly moving cells are likely macrophages (Gustafsson et al., 2001). Voxel size 0.69x0.69x4.13µm.



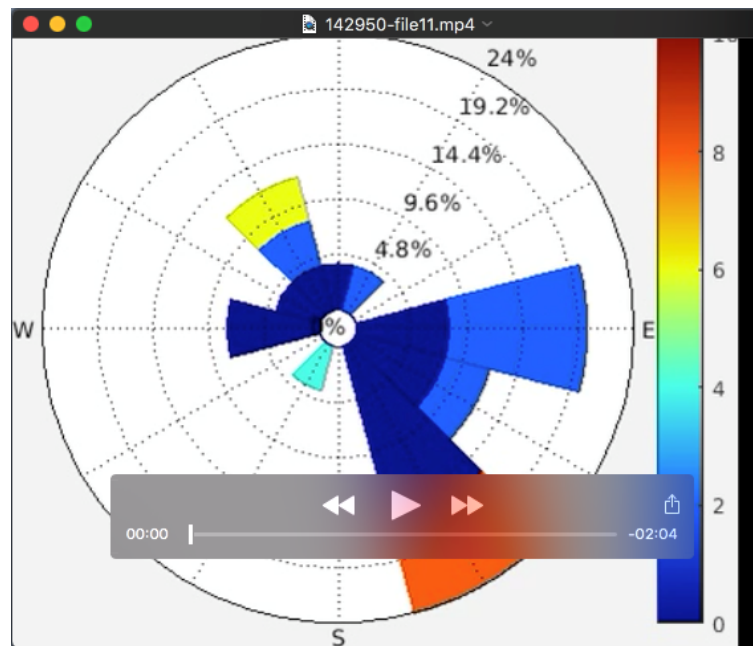
Movie 2. Branching of the UB in the FiZD culture. The embryonic kidneys from E11.5 *mTmG; Hoxb7Cre* embryos were dissected and cultured in the FiZD. Organogenesis was monitored for 5 days. The generated movie illustrates the *Hoxb7Cre* induced GFP expression in the cells of the ureteric bud. A stack of 20 z-layers was captured using a 20x/0.8 M27 Zeiss Plan-Apochromat objective, images were acquired every 5 minutes, Voxel size 0.69x0.69x1.82µm.



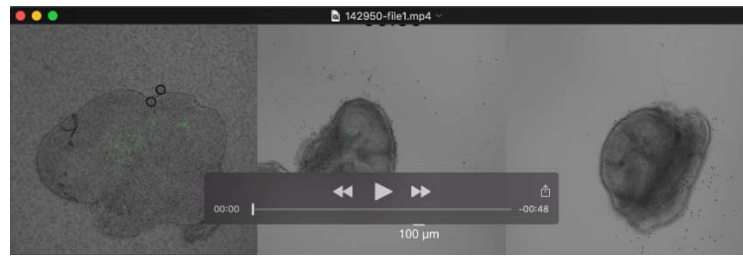
Movie 3. The FiZD time-lapse of kidney organoid. The kidneys of the E11.5 *mT/mG;Wn4Cre* embryos were dissected, the metanephric mesenchyme (MM) was separated and subjected to dissociation and reaggregation and BIO mediated induction of nephrogenesis. The nephron precursor cell derived structures are GFP+ due to *Wnt4Cre* mediated activation of the floxed *R26R GFP* reporter. Note that the FiZD culture set up enables tracking of the nephrogenesis process at the single cell resolution in the time-lapse set up. The nephrogenesis was monitored for ten days. The period of time of first 4 days is represented. 10 z-layers were captured; images were acquired every 15 minutes 20x/0.8 M27 Zeiss Plan-Apochromat objective, voxel size 0.69x0.69x1.82µm.



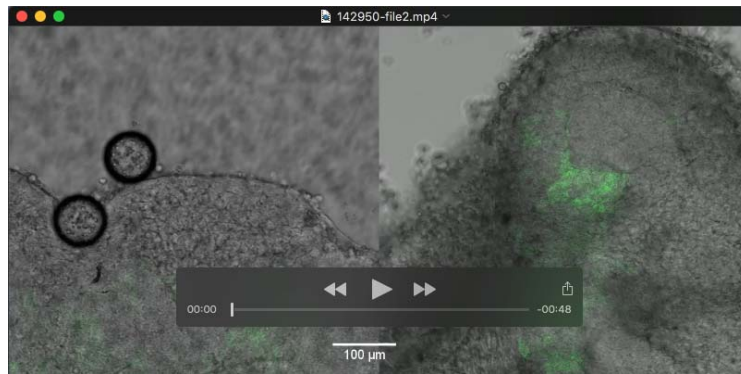
Movie 4. Computer-assisted cell segmentation and tracking for the FiZD derived image data. The quality of the FiZD captured images is good enough for computer assisted analysis of the renal cell behaviour such cell movement during morphogenesis. The movie shows the movement of kidney cells within the ureteric bud branch tip. Green line marks the separation between kidney stem and branch tip. Red line splits the ureteric bud branch tip in the middle to enable the analysis of movement of cells in both halves of the branch tip. Individual cell boundaries and their tracks are also shown.



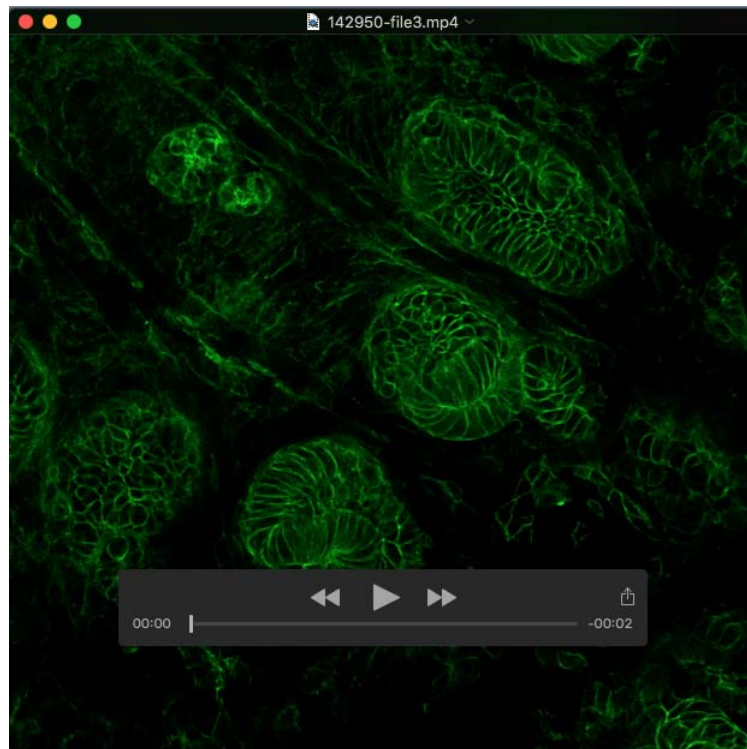
Movie 5. Computer assisted analysis of renal cell migration dynamics can be achieved with the FiZD. Subjection of the time-lapse image stack from the FiZD to computer aided image analysis enabled quantitation of the cell migration dynamics as depicted with the Wind rose plot. The Wind rose plot shows the distribution of magnitude and direction of the cell movements in right half of ureteric bud branch tip for each time point. Length of spokes indicates the proportion of cells moving in a particular direction and thickness of colour bands within a spoke indicates the distribution of speed for these cells.



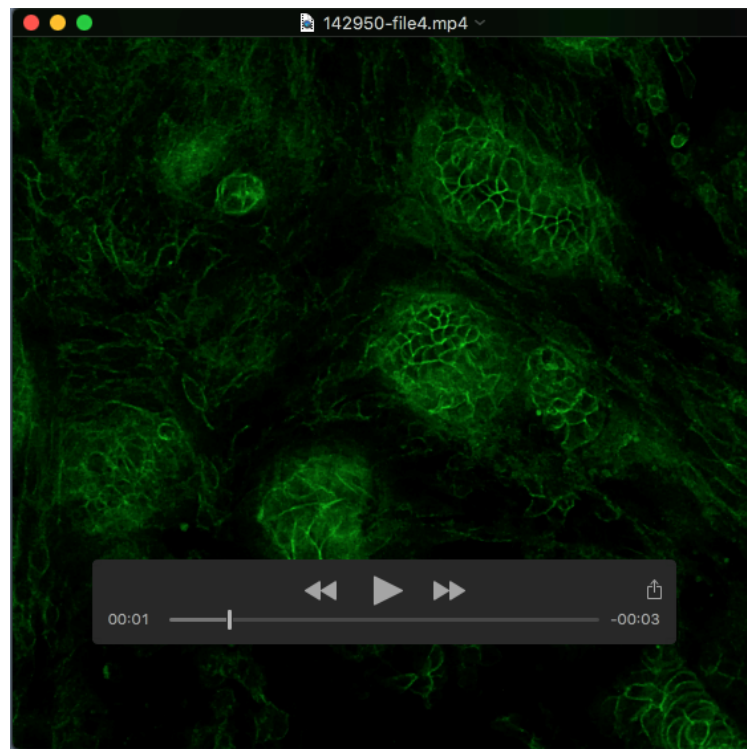
Movie 6. Comparison of low-volume and the FiZD culture systems using intact *ex vivo* embryonic kidney samples. Intact E12.5 kidneys of *Wn4Cre; mT/mG* embryos were placed in both culture systems and grown in time-lapse for 9 days. Left: FiZD, Middle and right: Low-volume method. Note “shrinking” of the kidney cultures in low-volume set up as a result of changing of culture medium (time points: 14:00, 38:00, 59:00, 77:00, 100:00 and 123:00). Note also moving of the sample at right panel out of the field of view. Two beads used as spacers can be seen on left panel. Images were taken every 15 minutes. One out of 20 Z-slices and period of time of 6 days are represented. A 10x/0.45 Zeiss Plan-Apochromat objective was used. Voxel size 0.69x0.69x4.13µm.



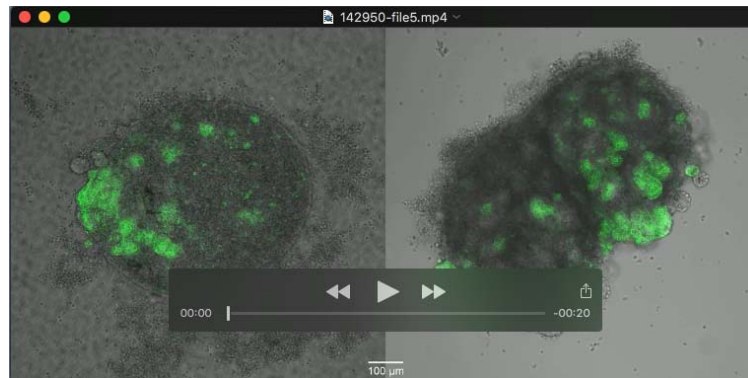
Movie 7. Close up from Movie 6 presenting the developing nephrons in both culture systems. Development of few nephrons is shown zoomed in to actual resolution. Left: FiZD and right: low-volume method. All other settings are as in Movie 6.



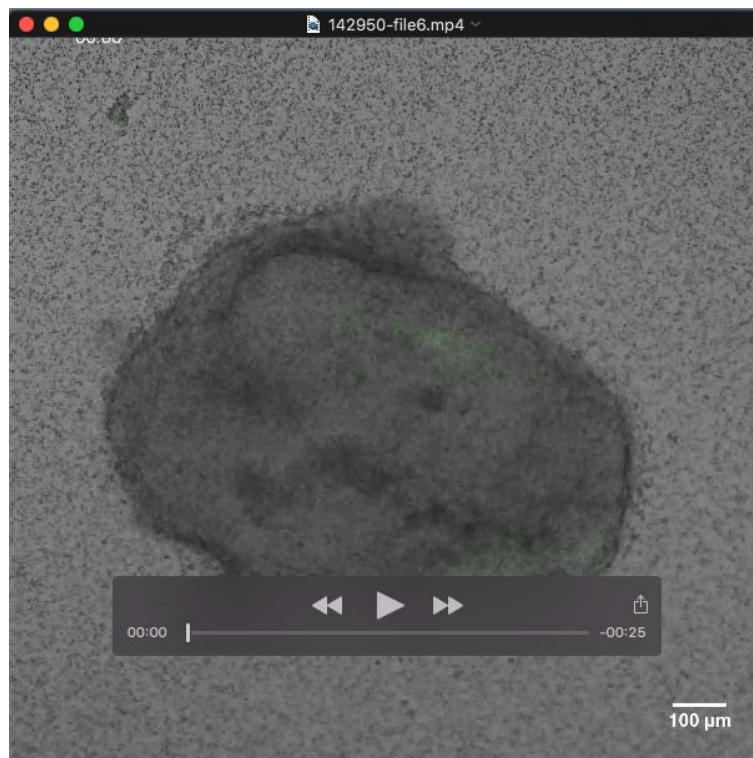
Movie 8. High resolution short time-lapse of developing nephrons. Intact E12.5 kidneys of *mT/mG; Wn4Cre* embryos were set up in FiZD culture. Images were taken every 5 minutes for 20 time points. A stack of 53 Z-layers was acquired and a single optical plane is represented. A 25x/0.8 Zeiss LCI Plan-Neofluar water immersion objective was used. Voxel size 0.17x0.17x1.13 μ m.



Movie 9. Scan through all the z-layers during single time point in the same time-lapse experiment as represented in Movie 8. The developing nephron was imaged in time-lapse experiment (see Movie 8). Complete Z-stack of all 53 Z-slices is shown. Other settings are the same as in Movie 8



Movie 10. Comparison of silicon chamber and FiZD systems with an embryonic kidney organoid. Kidney organoids prepared from E13.5 frozen embryonic kidneys were placed in both culture systems and grown in time-lapse experiment for 3 days. Images were taken every 15 minutes. A stack of 20 z-layers was captured using a 10x/0.45 Zeiss Plan-Apochromat objective, voxel size 0.69x0.69x4.13µm.



Movie 11. *mTmG;Wnt4Cre* embryonic kidneys cultured on top of Trowell insert. E12.5 kidneys cultured for 3 days on top of the Trowell insert. A stack of 20 z-layers was captured using a 10x/0.45 Zeiss Plan-Apochromat objective, images acquired every 15min. Voxel size 0.69x0.69x4.13μm.

Supplementary figures

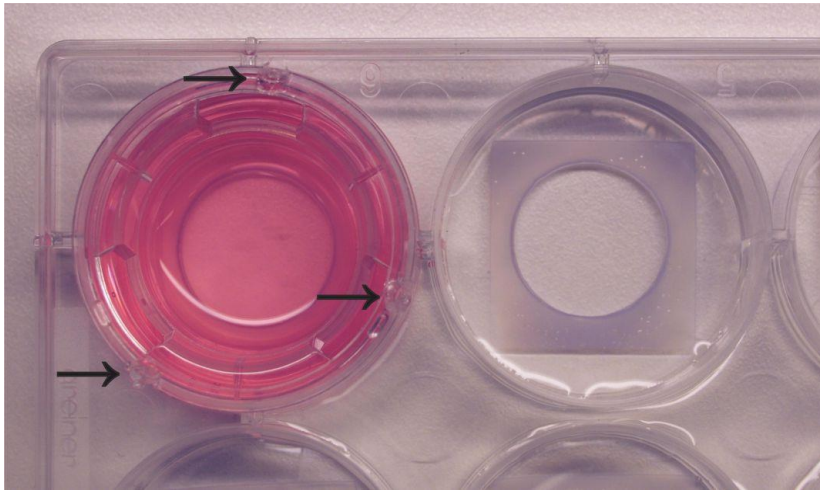


Fig. S1. Preparation of the plate for the FiZD culture.

FiZD is prepared in 6-well CellStar plates (Greiner bio-one) using Transwell inserts (24mm Transwell® with a 0.4µm-pore polyester membrane). A round hole (20 mm diameter) was drilled at the bottom of each well and glass coverslips (24x24 mm) were glued at the upper site of the bottom with using either dental wax or Histoacryl® glue (Braun Ref 1050052). The cover slips need to be cleaned with the protocol presented further in the Supplemental data. This promotes sticking of the organ rudiments to the cover slip glass. Prior to setting up the FiZD culture the processed plates were rinsed with ethanol, distilled water and then dried in a UV hood. Transwell insert is shown placed in the well 1 and the points on the rim where the insert is melted and fixed to the plate are shown with arrows.

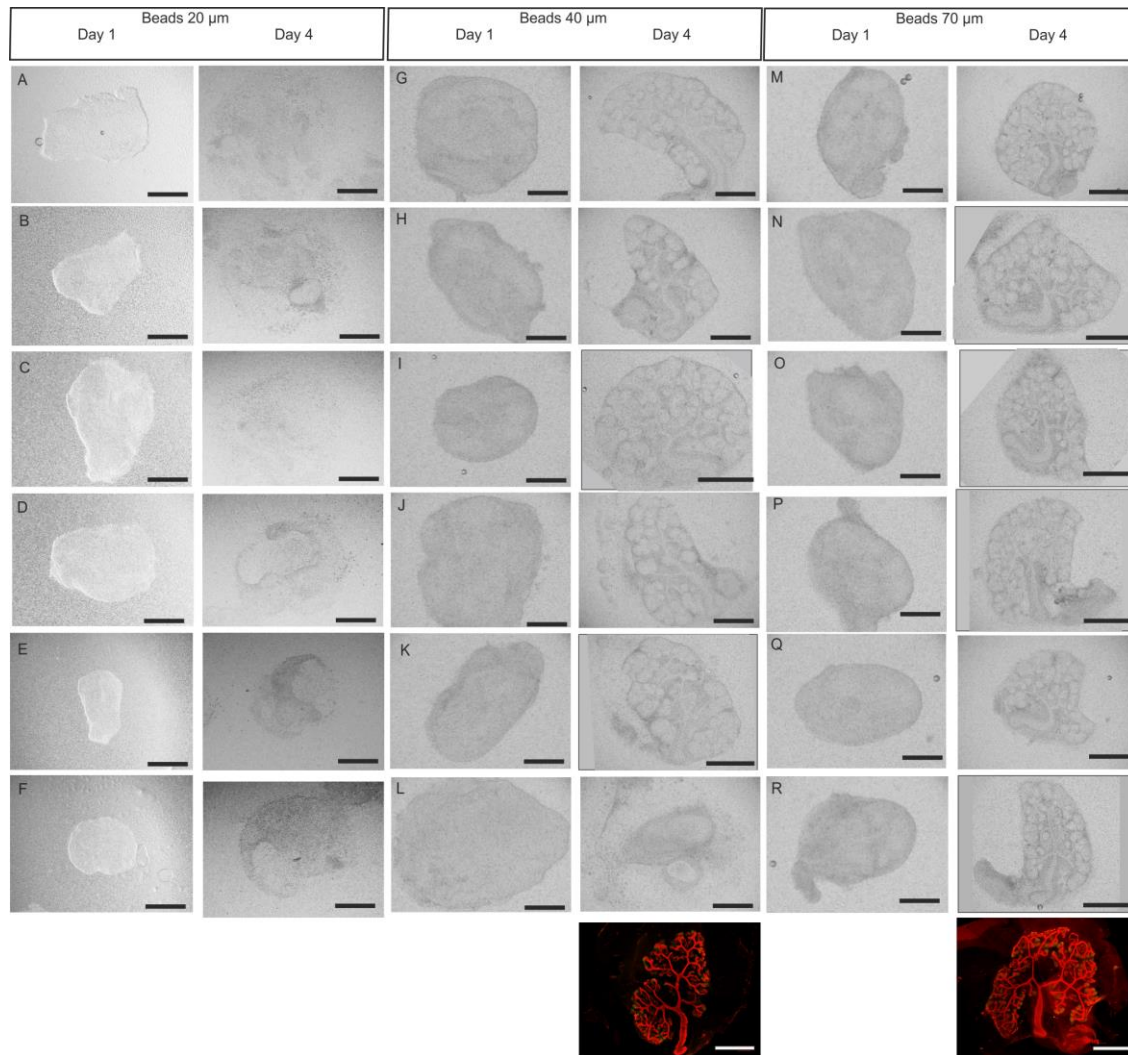


Fig. S2. Comparison of the effect of spacer beads size on the culture. Embryonic kidneys were cultured in planar organ culture with different spacer bead sizes 20, 40, and 70 μm . If the 20 μm beads are used (A-F) the development is ceasing after second day. In the 40 (G-L) and 70 (M-R) μm cases the kidney development is able to proceed, but when using the 40 μm beads there is more variability (L).

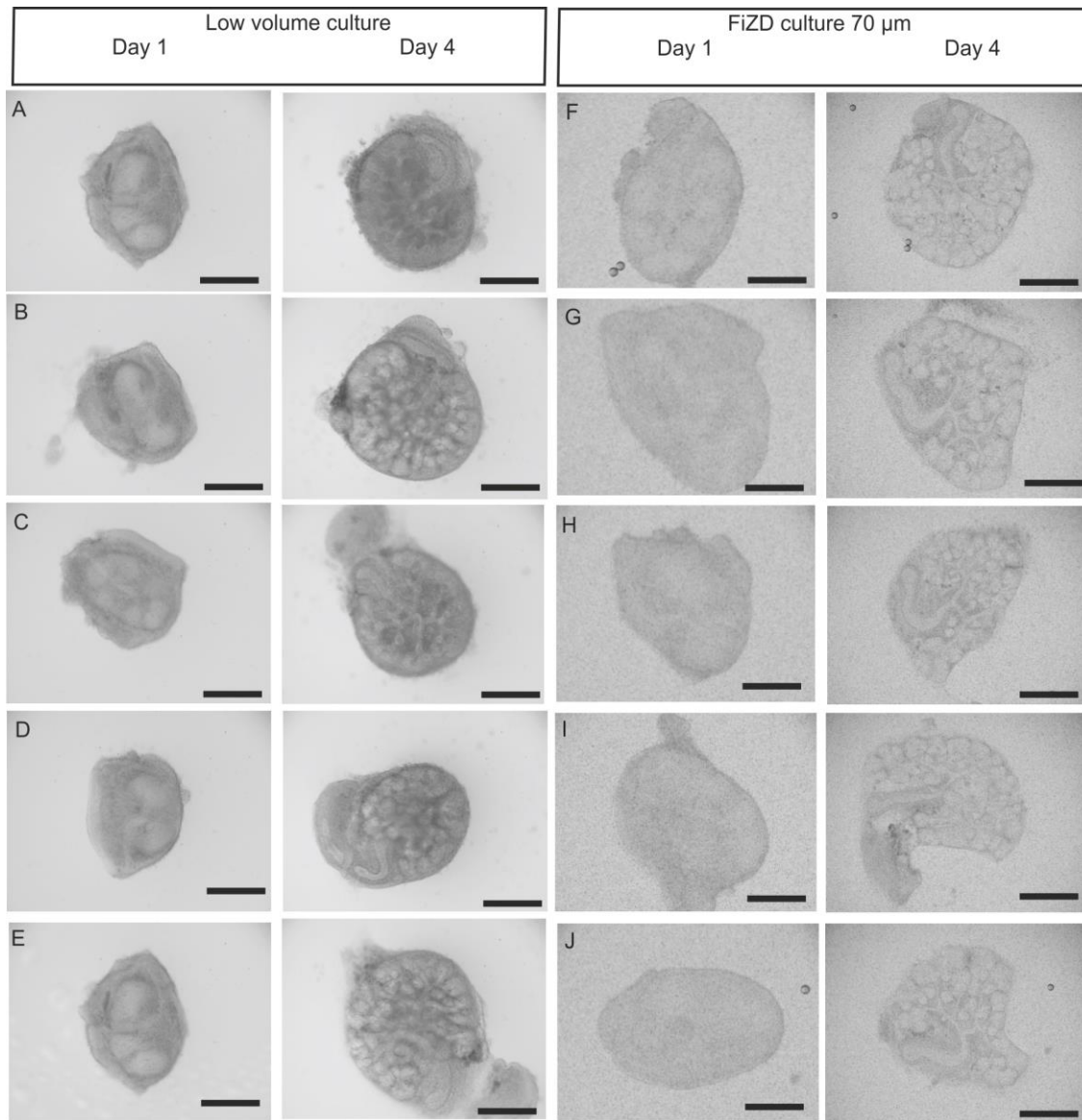


Fig. S3. Comparison of the Low-volume and FiZD methods. Embryonic kidneys were cultured in both Low-volume culture (A-E) and FiZD (F-J) for 4 days. The images show that the development proceeds well in both of the cases. In the FiZD culture the internal morphology of the developing kidneys is more clearly visible.

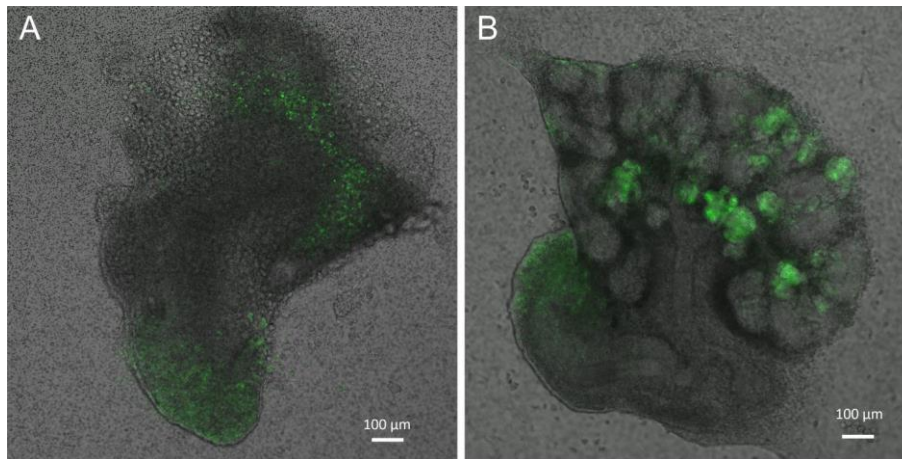


Fig. S4. The effect of increased laser power on the viability of the embryonic kidneys in the Movie 11. Embryonic kidneys were placed on top of a Transwell insert and one of them (A) was imaged for 3 days. The laser power had to be doubled from the FiZD culture settings because the kidney is further away from the objective and located on top of the filter. The development of the kidney was seriously affected when compared to the not imaged (B). A stack of 20 z-layers was captured using a 10x/0.45 Zeiss Plan-Apochromat objective, images acquired every 15min.

Supplementary methods

Segmentation of the image data

The middle slices of the confocal data stacks were used for UB cell tracking. The centroid of the segmented cells in the first frame was used to initialize cell tracks. Hungarian algorithm was used to associate cell tracks in a frame with detections in the next frame. Cell detections which remain nonassociated were used to initialize new tracks. Once all frames had been processed, errors in cell tracking due to missing detections were corrected by associating cell tracks ending at a frame with other cell tracks beginning in the next 3 frames using Hungarian algorithm. The data obtained from the time-lapse cultures is in 3D but the analysis was performed on the 2D slices of the image stacks. The lower resolution in axial direction compared to resolution within a slice, and the blurring

in axial direction due to chromatic and spherical aberrations made the analysis of data in 3D more challenging.

Wind Rose Plot:

1) The speed and direction of movement for all cells in a frame are computed. 2) Cells are placed into 12 bins (directions) according to their movement direction. 3) For each of the 12 directions, a spoke is drawn. The length of this spoke indicates the proportion of cells moving in the direction covered by the arc of that spoke (in these Wind rose plots, the size of all arcs is 30 degrees, only their direction differs). The circles (circle labels [3.75, 7.5, 11.25, and 15] are shown in the Wind rose plot) can be used to quickly estimate what proportion of cells is moving in a particular direction. 4) All cells within each spoke are placed into 8 bins according to their speed. These bins are shown with different colours (dark blue means cells are moving very slowly with a speed between 0 and 0.2 $\mu\text{m}/\text{min}$ and dark brown means cells are moving with speed greater than 1.4 $\mu\text{m}/\text{min}$, the speed for other colours can be checked from the scale on the right side).

If a spoke has only 1 colour e.g. dark blue, it means that all cells moving in that direction have speed less than 0.2 $\mu\text{m}/\text{min}$. Cells with the lowest speed are drawn first, i.e. the dark blue colour band is drawn first and is closest to circle centre than other colour bands and dark brown colour band is drawn last and is furthest from circle centre. Wind rose plots also show that most cells are moving very slowly (thickness of dark blue colour bands is much more than thickness of other colour bands).

Preparation of the cover slips for FiZD culture

1. Heat cover slips in a loosely covered glass beaker in 1M HCl at 50-60°C for 4-16h.
2. Cool to room temperature
3. Rinse out with 1M HCl with ddH₂O

4. Fill the container with ddH₂O and sonication in water bath for 30 minutes, repeat
5. Fill container with 50% EtOH and 50% ddH₂O and sonicate in water bath for 30 minutes
6. Fill a container with 70% EtOH and 30% ddH₂O and sonicate in water a bath for 30 minutes
7. Fill container with 95% EtOH and 5% ddH₂O and sonicate in water bath for 30 minutes
8. Fill a container with 95% EtOH .
9. Transfer the cover slips into a box with Whatman filter and keep for autoclaving.