Optical projection tomography imaging to study kidney organogenesis

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Abstract
Optical projection tomography (OPT) is a 3D imaging technology. The 3D tomographic reconstruction permits precise analysis and quantification of various structures in developing embryonic tissues, adult organs of small rodents or biopsies. OPT enables detailed and accurate studies of kidney organogenesis, namely ureteric tree branching morphogenesis and nephron quantification.

Key words: Optical projection tomography, phenotyping, 3D imaging, branching morphology, nephron quantification, whole mount immunostaining, whole mount *in situ* hybridization.

1 Introduction

Optical projection tomography (OPT) is a three-dimensional (3D) imaging technology. It allows rapid 3D imaging of mesoscopic (1mm to 10 mm) specimens that have fluorescent and non-fluorescent signals (1). This technique has made a significant impact in the field of developmental biology. OPT permits acquisition of 3D digital data, which is used for computational analysis of multiple parameters such as developing kidney ureteric tree branch length, angles or nephron distribution and counts.
Prior the OPT scan the specimen is stained and embedded to an agarose gel which serves as a support during imaging. The clearing agents are used to optically clear the specimen. This will enable light passage through the tissue with minimal scattering, absorption and reflection. Cleared specimen is attached to a magnetic holder and steadily rotated 360 degrees around a single axis. A digital image is taken at every chosen angular step (0.45-0.9 degrees). After image acquisition, virtual sections are reconstructed by using a back-projection algorithm. The high-resolution images can be viewed and analyzed as tomographic sections and as a whole.
Here we describe detailed step-by-step protocols for mouse embryonic kidney preparation for OPT after whole mount immunostaining, whole mount *in situ* hybridization (WISH) or both techniques combined.
Whole mount immunostaining permits antibody staining and protein expression detection in entire embryo or tissue. The WISH is using labeled complimentary RNA to localize specific RNA sequence in the tissue. There are numerous WISH protocols, here we will describe an automated whole mount *in situ* hybridization procedure (2). Automated process allows completing the WISH within three days with moderate hands on time versus laborious five-day manual protocol. The automated WISH protocol can be done manually using the same material and procedure. It will also be described how to combine WISH and immunostaining techniques allowing simultaneous examination of protein and RNA expression in the same tissue.

2. Materials

2.1. Whole mount immunostaining

1. Phosphate Buffered Saline (PBS).
2. PBT-X-100: 1x phosphate buffered saline (PBS) with 0.1% Triton-X-100 added. Make fresh.
3. Paraformaldehyde (PFA) 4%: dissolve PFA powder in 1xPBS, in water bath at 65 ºC. Filter through 0.45 µm syringe filter.
4. Bleaching buffer: 15% H_2O_2 and 15% dimethyl sulfoxide (DMSO) dilute in absolute methanol and filter through 0.45 µm syringe filter.
5. Blocking solution: 5% DMSO, 10% of heat-inactivated serum, derived from the same species as secondary antibody, diluted in PBT-X-100. Filter through 0.45 µm syringe filter.

2.2.1. Whole mount *in situ* hybridization probe synthesis

1. RNAase free diethylpyrocarbonate (DEPC) treated water: 1/100 vol 10% DEPC in 100% EtOH add double distilled (dd) water and autoclave.
2. 5x transcription optimized buffer (Promega), comes with the RNA polymerase and contains DTT 100 mM (Promega).
3. Dig RNA labeling Mix 10X Conc (Roche).
4. RNAse inhibitor, Recombinant RNasin ® Ribonuclease Inhibitor 2500u, Promega.
5. Appropriate RNA polymerase: SP6, T3 or T7.
6. DNAse I (Promega).
7. 4 M LiCl: dissolve in DEPC water and autoclave.
8. 70% Ethanol diluted in DEPC water.

2.2.2. Whole mount *in situ* hybridization

1. Diethylpyrocarbonate (DEPC) treated water made as in 2.2.1 item 1.
2. Phosphate Buffered Saline (PBS).
3. PBT-20: 1x PBS with 0.1% Tween20. Make fresh.
4. Hybridization buffer: 50% ultra-pure formamide, 5x SSC (20x SSC stock solution: 175 g NaCl, 88.2 g Na citrate, set pH to 4.5, add DEPC water up to 1L.), 50 µg/ml of tRNA, 1% SDS, 50 µg/ml heparin sodium salt Grade I-A and DEPC water. Buffer can be prepared in advance and stored at -20 ºC for a month.
5. Proteinase K stock solution: 10 mg/ml dissolved in sterile DEPC water, store at -20 ºC. Working solution 0.01 mg/ml diluted in PBT-20.
6. Paraformaldehyde (PFA) 4%: dissolve PFA powder in 1x PBS prepared in DEPC treated water, in water bath at 65 ºC.
7. Glycine 0.2%: dissolve in PBT-20, make fresh before use and filter through 0.45 µm syringe filter.
8. Hydrogen peroxide 6%: dilute in DEPC water.
9. Washing buffer I: 25 ml formamide ≥99%; 12.5 20x SSC, pH 4.5 (made as in 2.2.2. item 4 but use dd water instead of DEPC), 5 ml 10% SDS, 7,5 ml dd water.
10. Washing buffer II: 25ml formamide ≥99%; 5 ml 20x SSC (made as in 2.2.2. item 4 but use dd water instead of DEPC), 1ml 10% SDS, 19 ml DEPC water.
11. Maleic acid buffer (MAB): 100mM maleic acid, 150mM NaCl, pH 7.5.
12. MABT: Maleic acid buffer (MAB) with 0.1% of Tween20. Add Tween20 just before use.

13. Blocking solution: 0.5% blocking reagent (Roche) dissolved in MAB according manufacturers recommendations, supplement with 10% heat inactivated sheep serum.
14. Antibody and antibody dilution buffer: 0.5% Blocking reagent (same as in item 13, Roche) dissolved in MAB, supplemented with 1% heat inactivated sheep serum and add anti-Digoxigenin-AP Fab (Roche) diluted 1:1000 (1.5 U/ml).
15. NTMT: 100mM NaCl, 100mM Tris, pH9.5, 50mM $MgCI_2$, 1% Tween20.
16. BM Purple AP Substrate precipitating solution (Roche).
17. InsituPro device from Intavis AG Bioanalytical Instruments or equivalent.

2.3. Preparation for OPT scanning

1. A 5 ml syringe.
2. Regular or low melting point agar.
3. Clearance solution BABB: Benzyl alcohol and Benzyl benzoate solution dilution ratio 1:2.
4. Clearance solution scaleCUBIC-1 reagent (3): 25wt% urea, 25wt% N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine and 15 wt% Triton X-100.
5. Super glue (Loctite).
6. Optical Projection Tomography scanner Bioptonics 3001 (Bioptonics) or equivalent.
7. Image reconstruction software nRecon (Skyscan Pty Ltd., Kontich, Belgium).
8. 3D analysis software: open access, Fiji, Drishti, Vaa3D and Kidney Analysis Application (7); commercial, Imaris (Bitplane AG), Amira (Visage Imaging Inc) and Volocity (Perkin Elmer).

3. Methods

3.1. Whole mount immunostaining

1. Dissect mouse embryonic kidney or genital ridges, in cold PBS.
2. Fix tissues in ice-cold 4% PFA for 30 min- 1 h depending on mouse age and kidney size (E11.5-E13.5- 30 min, E14.5-E16.5 60 min), at 4 ºC.
3. Wash specimens twice for 10 minutes in PBS, to remove PFA.
4. These specimens are ready for whole mount immunostaining (see Note 1 for storing instructions).
5. Bleach specimens in 15% H_2O_2 and 15% DMSO diluted in absolute methanol at room temperature for 12 h while rocking.
6. Wash specimen in PBST-X-100 three times for 20 min, while rocking.
7. Block the tissues in blocking solution containing 10% serum, 5% DMSO in PBST-X-100 (see Note 2). To ensure good blocking use the serum from the same species where the secondary antibody was derived. Incubate for 12 h at room temperature, while rocking.
8. Dilute primary antibodies in blocking solution and incubate for 24 h at room temperature, while rocking.
9. Wash in PBST-X-100 for 12 h, change to fresh PBST-X-100 for six times every 2 h.
10. Dilute secondary antibodies in blocking solution and incubate for 24 h at room temperature, while rocking.
11. Wash in PBST-X-100 for 12 h, change the solution for five times.
12. Proceed to preparation for OPT scanning (3.4 Preparation for OPT scanning).

3.2. Whole mount *in situ* hybridization

We assume that the gene of interest was already subcloned to the vector (pBlueScript II or other) for *in situ* probe preparation. The optimal DNA length for RNA probe production is about 800 bp +/- 200 bp. It is important to prepare and run simultaneously anti-sense probes for positive signal and sense probes for negative control.

3.2.1. Probe synthesis

1. Mix reagents in the following order at room temperature: linearized plasmid 1 µg, 5x transcription buffer 4 µl, 10x DIG reaction mix 2 µl, 0.1 M DTT 2 µl, RNAse inhibitor 1 µl, polymerase 2 µl, DEPC water up to total reaction volume 20 µl.
2. Incubate for 2h at 37°C.
3. Remove one microliter of the mixture and save for electrophoresis gel.
4. Add 2 µl DNaseI to the rest of the probe synthesis reaction from the step 1.
5. Incubate for 15 min at 37°C.
6. Add 100 µl DEPC water, 10 µl 4M LiCl, 300 µl EtOH and mix. Incubate on dry ice or -70˚C for 20 min.
7. Spin in the tabletop centrifuge at 4°C, at 12000 g for 10 min. Discard the supernatant.
8. Add 200 µl of 70% EtOH (ethanol diluted with DEPC water), spin in the tabletop centrifuge at 4°C, at 12000 g for 10 min. Discard the supernatant.
9. Repeat step 8.
10. Invert the eppendoff tube and air-dry the pellet at room temperature for about 15 min.

11. Resuspend pellet in 45 µl DEPC water. Remove 1 µl of solution and save it for electrophoresis gel.
12. Add 45µl formamide to sample, mix well and store at -70°C. Probes can be stored at -70°C for several months without any damage.
13. Run electrophoresis gel with the samples from steps 3 and -11. Add 9 µl of 1x loading buffer to 1 µl of spared specimen and load on the agarose gel. In the specimen from the step 3 will be visible both DNA and RNA bands, whereas in the specimen from the step 11 only RNA band should be visible. If RNA band is visible, proceed to *in situ* hybridization. If not, repeat and optimize probe synthesis.

3.2.2. Whole mount *in situ* hybridization (WISH)

1. Dissect and fix embryonic kidney as in indicated in 3.1. item 1 and 2. Wash specimens twice in PBS for 10 minutes, 50% ethanol for 10 min and twice in 70% ethanol for 10 min. These specimens are ready for loading to *in situ* hybridization machine or they can be stored at -20°C for several months (see Note 1).
2. Place specimens to InsituPro machine columns (comes with the device) filled with 70% ethanol. Use medium incubation columns, with outlet needles. If specimens are very small, use column inserts. Device can process thirty columns at a time and multiple specimens per column (Fig.1). (see note 3 for manual WISH).

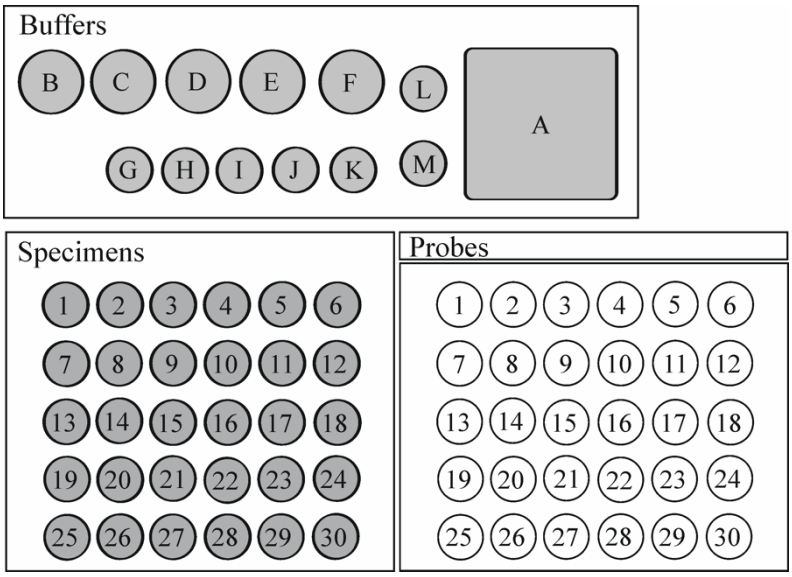

Fig. 1 Schematic representation of buffer, specimen, and probe locations for automated WISH in InsituPro (Intavis AG Bioanalytical Instruments) device.

3. Prepare buffers for Day I (Table 1) and load them to the device in the order indicated in the Fig. 1.
4. Cut the caps of from PCR tubes. Pipette 0.45 ml of prewarmed hybridization buffer, add 2.5 µl of RNA probe (about 0.1 µg/µl). The probe concentration might need to be optimized to get best signal. Place tubes with probes in an order corresponding the specimen order (Fig. 1). Robotic arm will pipet

preheated probe on the specimen during the first day. The hybridization temperature generally ranges from 60-70 ºC. It might need to be optimized for each probe individually.
5. Set the program for InsituPro device to run following the manufacturer's instructions. An example for the program is presented in the Table 2.
6. Based on this program device will stop twice. During the first stop load buffers for Day II and during the second stop load buffers for Day III as in the order indicated in the Table 1.
7. On Day III, once device has stopped, remove specimens from columns and place to a 24 well plate.
8. Add 400 µl (or enough to cover the specimen) of BMP purple AP substrate precipitation solution. Wrap to aluminum foil to protect from light. Incubate at room temperature and follow color developing every 30 min. If color dose not develop extend incubation over night at +4C.
9. As soon as the color is strong and background remains clear, stop reaction by washing in PBT-20 three times for 15 min.
10. Post-fix in 4% PFA for 1 h at room temperature.
11. Wash in PBS three times for 10 min.
12. Proceed to preparation for OPT scanning (3.4 Preparation for OPT scanning).

3.3. Combined whole mount immunostaining and whole mount *in situ* hybridization

1. First, follow the whole mount *in situ* hybridization protocol 3.2.2. after completion wash specimens in PBS twice for 10 min.
2. Proceed with whole mount immunostaining with antibodies of choice as indicated in section 3.1.
3. Once both staining will be completed proceed to preparation for OPT scanning 3.4.

3.4. Preparation for OPT scanning

We will refer to Bioptonics 3001 device, there are other open OPT platforms available (4, 5, 6) and the staining and preparation steps are equally suitable for all.
1. Observe the specimen in bright and fluorescence light under microscope and remove all possible impurities adhered to the surface of the specimen.

2. Prepare 1% of regular or low melting point agarose dissolved in purified water and filtered (see Note 4).

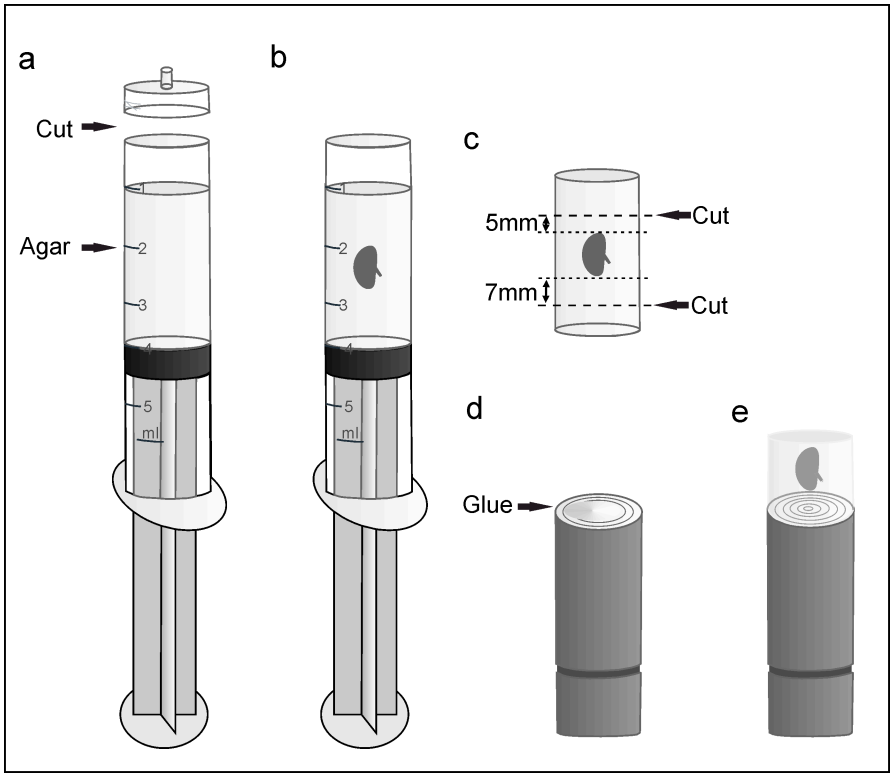

Fig. 2 Tissue embedding to agar and mounting for OPT imaging. Cut the tip of syringe and pour agar (a). Place the specimen in, and move it around to position to the center as gel casts (b). Let the gel set, plunge the gel cylinder out from the syringe, and trim (c). Apply glue to the magnetic holder (d), and place the specimen (e)

3. Cut the tip of 5 ml syringe (Fig. 2a), secure the syringe to stand, put the plunger at 4 ml mark, pour 3 ml of the melted agar in (Fig. 2a) and put in the specimen (Fig. 2b). Keep on swirling specimen around with the plastic sticks or tweezers to position it to the center of the syringe as the gel is solidifying (Fig. 2b). Avoid bubbles. Let it set for 15 min at 4 ^{o}C.
4. Remove gel cylinder from syringe by plunging it out (Fig. 2c).
5. Trim gel cylinder by cutting excess gel. Leave 7 mm gap between specimen and bottom. This will ensure that there is enough working distance and the magnetic holder will not be visible during imaging. Leave 5 mm gap between specimen and the top cut (Fig. 2c).
6. Clear the specimen in BABB (3.4. item 7) or ScaleCUBIC-1 (3.4. item 8).
7. Benzyl alcohol: Benzyl benzoate solution (BA:BB, 1:2). Prior BABB clearance dehydrate tissue in 50% ethanol for 2 h, absolute ethanol 6 h and fresh absolute ethanol for 6 h. Add BABB and incubate for 12 h. Change to fresh BABB after 12 h and continue clearance for another 12 h or as long as specimen will become transparent (See Note 5 and 6).
8. ScaleCUBIC-1 reagent clearance method will take longer time but will not require tissue dehydration. Change ScaleCUBIC-1 reagent after 24 h and continue clearance until specimen will become transparent (see Note 5).

9. Once specimen is cleared, remove it from the solution and slightly dry the bottom side on a paper. Apply glue on the magnetic holder (Fig. 2d) and place the specimen on the top (Fig. 2e). Let it set for about 15min.
10. Prepare OPT device for scanning following the appropriate instructions.
11. Place the prepared specimen into the device and scan it in clearance solution. Choose the correct acquisitions channels: fluorescence or/and transmission. On Bioptonics 3001 device, set camera resolution to high (1,024 x 1,024), rotation steps to 0.45° and the zoom to fit specimen to the field of view at all rotational angles. The specimen will rotate 360° and device will acquire 800 images.
12. Reconstruct scanned images to *z*-series to acquire OPT tomographic data by using nRecon software.
13. Proceed to analysis by your favorite software (software list is in 2.3. item 8).
14. An example of double immuno-labeled mouse kidney at E16.5 with anti-Troma (grey) and anti-Nephrin (red) antibodies (Fig. 3). The software-assisted quantification of nephrons is rather rapid and accurate (Fig. 4), in this case we have used Spots detection feature available on Imaris software.
15. An example of terminal tip quantification. We have used WISH method and RNR probe (*Wnt11,* depicted in red) to detect and quantify terminal tips of the developing ureteric tree in E15.5 mouse kidney and quantified them using Spots detection feature available on Imaris software (Fig.5).
16. An example of ureteric tree branching analysis in developing mouse kidney at E15.5 by filament tracing function (Imaris) (Fig. 6). Analysis permits to acquire multiple parameters such as branching angles, number of terminal tips, branch length and oth. Kidney were whole mount immunostained with anti-Troma antibody (grey).

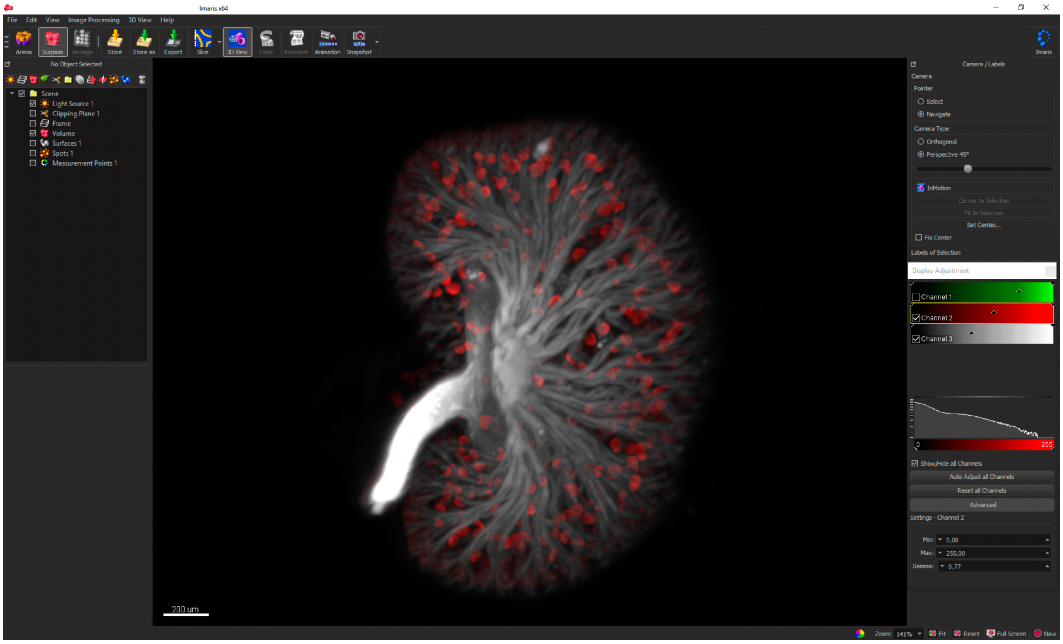

Fig. 3 Representation of E16.5 mouse kidney double immuno-labeled with anti-Troma (gray) depicting ureteric tree and anti-Nephrin (red) antibodies showing nephrons. 3D visualization was done by Imaris program

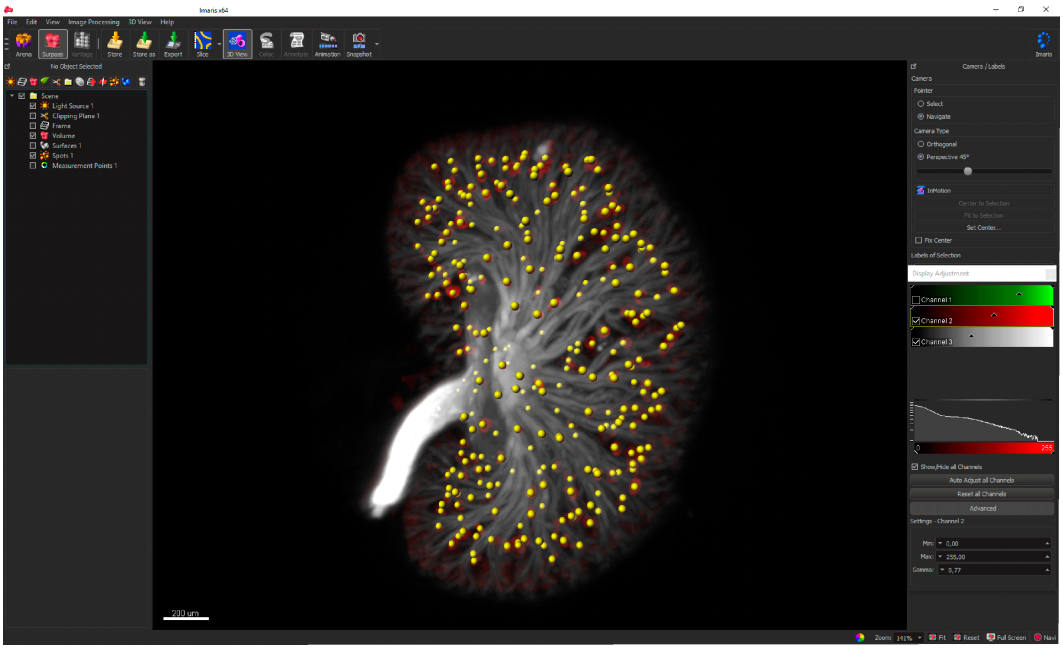

Fig. 4 Example of automated nephron quantification by spot detection function (Imaris). Nephrons are depicted in yellow following nephrin staining (red)

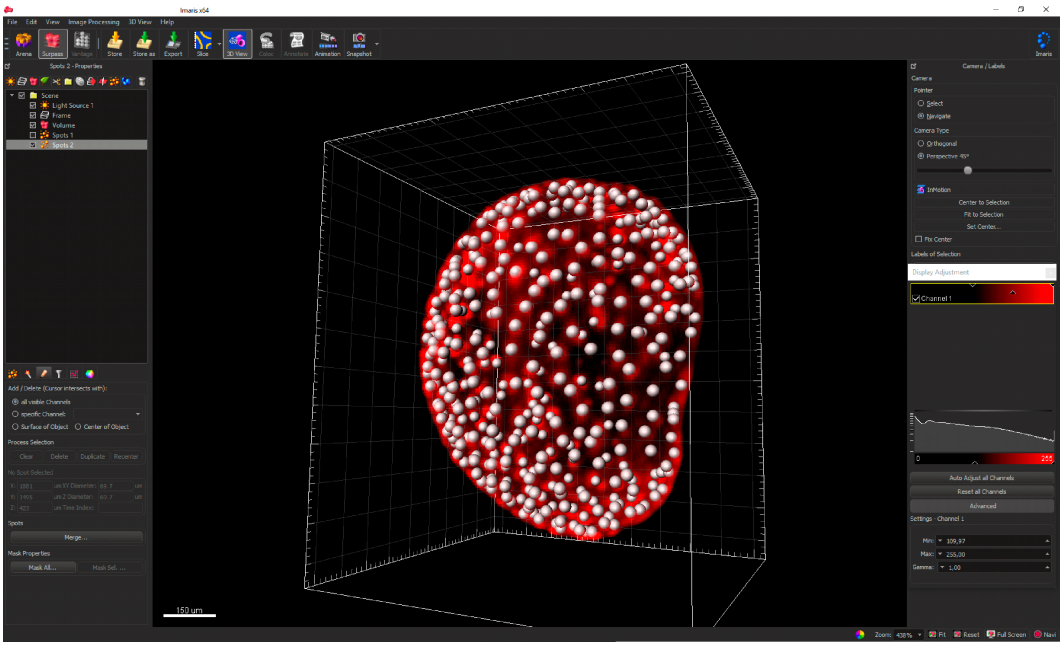

Fig. 5 Ureteric bud (UB) tip quantification by spot detection function (Imaris). UB tips labeled with Wnt11 probe (red) by WISH in mouse kidney at E15.5

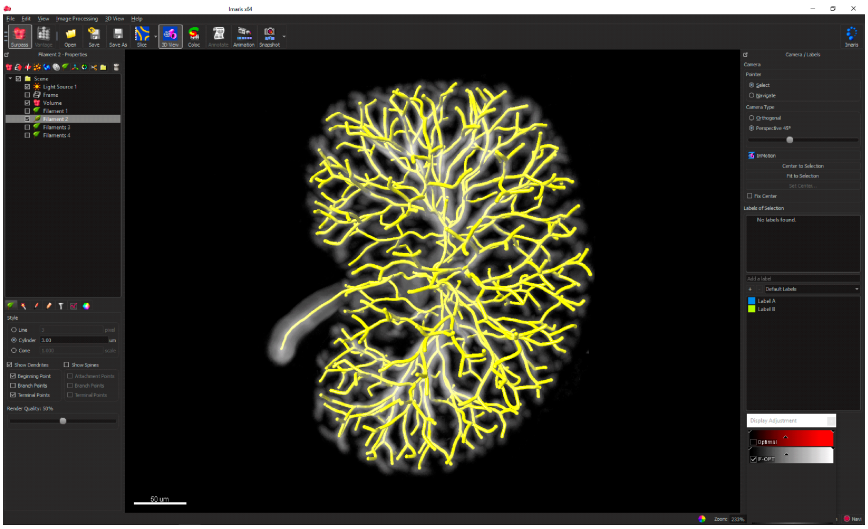

Fig. 6 Ureteric tree analysis in developing mouse kidney at E15.5 by filament tracing function (Imaris). Kidney stained with anti-Troma antibody (gray)

Notes

1. For storing, dehydrate specimens in 50% ethanol once for 10 min and twice in 70% ethanol for 10 min. Tissues can be stored in 70% ethanol at -20 ºC for several months. If specimens were stored in -70% ethanol Rehydrate them via ethanol series (70%, 50% and 30%) to PBS before starting immunostaining.
2. In order to prevent bacteria growth during long incubation time, supplement buffers with 0.01% sodium azide.
3. The same protocol can be used to do WISH manually. Place specimens to 24 well plates for washing steps and move them to glass bottles or 2ml eppendorff tubes for per-hybridization, hybridization and post-hybridization. Prepare all buffers as indicated and follow the procedure and incubation times indicated in the Table 2.
4. Use low melting point agarose (LMP) in cases when it is planned to reuse specimen after scanning for other applications. Other vice, regular agarose can be used. In order to remove specimen form LMP agarose rehydrate it from BABB to absolute ethanol for 6h, change to fresh ethanol for 6h, place to 50% ethanol for 6h and PBS for 6h. Put agarose block in PBS and warm up in water bath at 60 ºC. Once gel softens, gently retrieve the specimen.
5. The BABB clearance is rather rapid and suits very well for immunostained specimens. In case of whole mount *in situ* hybridization, BABB will gradually quench the color. The color will remain intact for about a week in BABB. In case of ScaleCUBIC-1 quenching is not an issue, but clearance will take longer time.
6. When working with BABB solution use appropriate protection, and handle solution in the fume hood. Use glassware as BABB is toxic and melts plastic.

5. References

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Table 1. The day-by-day work flow, buffer locations and amounts for automated WISH adopted for InsituPro.

Position	Buffers			Buffer amount for 30 samples
	Day I	**Day II**	**Day III**	
A	PBT-20	MABT		100 ml/120 ml
B	EtOH 50%			25 ml
C	Hybridization buffer			25ml
D		Washing buffer I		35 ml
E		Washing buffer II		35 ml
F		MABT		35 ml
G	Proteinase K			12 ml
H	4% PFA			12 ml
I	Glycine			12 ml
J	6% H2O2			12 ml
K			NTMT	12 ml
L		Blocking solution		12 ml
M		Anti-Dig antibody		12 ml

Table 2. Detailed program description for automated WISH adopted for InsituPro (Intavis AG Bioanalytical Instruments) device.

Nr.	Device action	Volume/ specimen	Time	Position/Buffer	Comments
1	Set temperature regulation	OFF			
2	Rinse	5000 µl			System wash
3	Aliquot	200 µl		B / Ethanol	Rehydration
4	Wait		5 min		
5	Incubate	350 µl	5 min	B / Ethanol	Rehydration
6	Incubate	200 µl	0 min		
7	Aliquot	200 µl		A / PBT-20	Rehydration
8	Wait		5 min		
9	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
10	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
11	Incubate	350 µl	1 h	J / 6% H2O2	Bleaching
12	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
13	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
14	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
15	Incubate	350 µl	35 min	G / ProtK	Tissue permeabilization
16	Incubate	350 µl	5 min	I / Glycine	Proteinase K inactivation
17	Incubate	350 µl	1 min	A / PBT-20	PBT-20 washes
18	Incubate	350 µl	35 min	H / 4% PFA	Post fixation
19	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
20	Incubate	350 µl	15 min	A / PBT-20	PBT-20 washes
21	Incubate	350 µl	15 min	C/ Hybridization buffer	Wash
22	Set temperature regulation	T2 (HIGH)			Set hybridization temperature (60-70 C)
23	Incubate	350 µl	1 h	C/ Hybridization buffer	Pre-hybridization
24	Incubate	350 µl	15 h	Probes	Hybridisation: robe will be pipetted on specimen
25	Wait for Key	350 µl			Device will stop; Load Day II buffers and continue
26	Incubate	350 µl	30 min	D / Washing buffer I	Post-hybridization washes I
27	Incubate	350 µl	30 min	D / Washing buffer I	Post-hybridization washes I
28	Incubate	350 µl	30 min	D / Washing buffer I	Post-hybridization washes I
29	Incubate	350 µl	30 min	E / Washing buffer II	Post-hybridization washes II
30	Incubate	350 µl	30 min	E / Washing buffer II	Post-hybridization washes II
31	Incubate	350 µl	30 min	E / Washing buffer II	Post-hybridization washes II
32	Set temperature regulation	OFF			
33	Wait		15 min		
34	Incubate	350 µl	15 min	F / MABT	MABT washes
35	Incubate	350 µl	15 min	F / MABT	MABT washes
36	Incubate	350 µl	15 min	F / MABT	MABT washes
37	Incubate	350 µl	15 min	A / MBT	MABT washes
38	Incubate	350 µl	1 h	L / Blocking solution	Blocking
39	Incubate	350 µl	6 h	M / Antibody	anti-Dig antibody staining
40	Incubate	350 µl	15 min	A / MBT	MABT washes
41	Incubate	350 µl	15 min	A / MBT	MABT washes
42	Incubate	350 µl	15 min	A / MBT	MABT washes
43	Incubate	350 µl	20 min	A / MBT	MABT washes
44	Incubate	350 µl	20 min	A / MBT	MABT washes
45	Incubate	350 µl	20 min	A / MBT	MABT washes

46	Incubate	350 µl	40 min	A / MBT	MABT washes
47	Incubate	350 µl	40 min	A / MBT	MABT washes
48	Incubate	350 µl	40 min	A / MBT	MABT washes
49	Incubate	350 µl	50 min	A / MBT	MABT washes
50	Wait for Key				Device will stop; Load Day III buffers and continue
51	Incubate	350 µl	15 min	K / NTMT	NTMT washes
52	Rinse	350 µl	5000 µl		
53	Set temperature regulation	350 µl	OFF		End of program

Figure legends

Fig. 1. Schematic representation of buffer, specimen and probe locations for automated WISH in InsituPro (Intavis AG Bioanalytical Instruments) device.

Fig. 2. Tissue embedding to agar and mounting for OPT imaging. Cut the tip of syringe and pour agar (a). Place the specimen in, move it around to position to the center as gel casts (b). Let gel set, plunge the gel cylinder out from the syringe and trim (c). Apply glue to the magnetic holder (d) and place the specimen (e).

Fig. 3. Representation of E16.5 mouse kidney double immuno-labeled with anti-Troma (grey) depicting ureteric tree and anti-Nephrin (red) antibodies showing nephrons. 3D visualization was done by Imaris program.

Fig. 4. Example of automated nephron quantification by Spot detection function (Imaris). Nephrons are depicted in yellow following Nephrin staining (red).

Fig. 5. Ureteric bud (UB) tip quantification by spot detection function (Imaris). UB tips labeled with Wnt11 probe (red) by WISH in mouse kidney at E15.5

Fig. 6. Ureteric tree analysis in developing mouse kidney at E15.5 by filament tracing function (Imaris). Kidney stained with anti-Troma antibody (grey).