**Visualization of the Primo Vascular System,**

**a Putative Cancer Metastasis Thread Afloat in a Lymph Duct**

Seung-Hwan Lee1 #, Sharon Jiyoon Jung1, 2 #, Kyoung-Hee Bae1\*, Hee Min Kwon3, Yoon Kyu Song2, Kwang-Sup Soh1\*

**Supplementary Information**

**2.1 REAGENT**

**Anesthesia:** Zolatil (Virbac Laboratories, Carros, France), Xylazine (Bayer, Korea).

**PVS staining:** Phosphate- buffered saline solution (PBS). 0.9% saline solution (Choongwae Pharmaceuticals, Korea).

**Contrast agent:**

AB staining: Alcian blue (AB) 8GX (Sigma, USA), PBS pH 7.2 (1X) (Life Technology Corp, USA).

PVS staining:Phosphate- buffered saline solution (PBS). 0.9% saline solution (Choongwae Pharmaceuticals, Korea).

**Histology:**

Phalloidin staining: Alexa Fluor 488 Phalloidin (Invitrogen, USA).

DAPI staining: 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen Molecular Probes, cat. no. D1306; 1:10,000).

**2.2. EQUIPMENTS**

**Microscopes and light source:** Stereomicroscope (SZX12, Olympus) with a CCD camera (DP70, Olympus, Japan), phase contrast microscope (BX51, Olympus, Japan) with a CCD camera (Infinity 3, Lumenera), confocal laser scanning microscope (CQ plus, Nikon, Japan).

Light source and optical fiber illuminator (Halogen lamp, KLS-100H-LS-150P, Kwangwoo Co, Ltd, Korea).

**Surgical instruments:** Surgical instruments and ophthalmic surgical instruments (Tumed, German) . Disposable Gentax latex glove (Geneall Biotechnology, Korea), Pet Specialty cordless trimmer (Oster, USA), masking tape (Scitech Korea Inc, Korea), gauze (Scitech Korea Inc, Korea), surgical drapes (Scitech Korea Inc. Korea), electric heating pad (size: 30 mm x 30 cm; Woojin Tech, Korea).

**Syringes and filters:** Hypodermic syringe (Kovax- Syringe, Korea), BD ultra-fine insulin syringe, 31G (Becton, Dickinson and Company, USA), BD 5- ml filter syringe (Becton Dickinson Medicals Ltd, Singapore), BD 10- ml filter syringe (Becton Dickinson Medicals Ltd, Singapore). Glass microfibre filters 110 mm (GE Healthcare Co., UK, cat. no. 1820-110), minisart syringe filter, hydrophilic (Sartoriou Stedim Biotech, Germany), BD Scalp Vein Set, 24G 3/4 (Becton, Dickinson and Company, USA), 3 IN Thin wall GL 1.5OD (World Precision Instruments, INC, USA, Item no. TW150F-3)

**Home-made syringe and instrument**: 3 IN Thin wall GL 1.5OD (World Precision Instruments, INC, USA, Item no. TW150F-3), Narishige puller (Narishige Group, Japan, Model no. PP-830)

**Staining and histology instruments:** pH meter (Thermo Electron Corporation, USA), glass funnel (Dongsung Science, Korea), round bottom test tube, 5 ml (BD Falcon, USA), coplin jar (Fischer Scientific, USA), pap pen (Invitrogen, USA), Vortex-2 Genie (Scientific Industries, USA). 5 –to-10- μl finnpipette (Lab Systems, Korea), disposable transfer pipette (Lappia, Korea). Micro slides (silane coating; size: 76 mm x 26 mm; Mutopure Chemicals Co, Ltd, Japan), 100 deckglaser cover slips (size: 24 mm x 50 mm; Knittel Glass, Germany), Leica CM1800 cryostat (Leica, Germany).

**2.3. REAGENT SETUP**

**2.3.1. Animals**

Spague Dawly (SD) rats (260~300 g, 9 weeks old) were used. Males are preferred as they develop less abdominal fat, making the surgery easier.

**2.3.2. Phosphate buffered saline solution (PBS) :** Eight (8) g of NaCl, 0.2 g of KCl 1.44 g of Na2HPO4, 0.24 g of KH2PO4 and 800 ml of distilled water are mixed together to make a solution, and using a pH meter, the pH is set to 7.4. An additional 200 ml of distilled water is added to the previously mixed solution to make 1 L (1000 ml) of 1x PBS solution, which is stored at room temperature.

**2.3.3. Alcian blue (AB) staining dye (1.0%**):

Combine 0.014 g of AB powder with boiled hot 7 ml of 1x PBS solution to make 0.2 % AB staining dye.

**STEPS**:

1. In order to inhibit the coagulation process of AB powder mix with the 1x PBS solution, we first boil the 10 ml of 1x PBS solution at high temperature (100 ˚C) until it reaches down to 7ml. Store this in a 10 ml falcon tube.
2. In maintained boil hot PBS solution, pour the 0.014 g of AB powder and mix these with continuous motion of gently inverting the tube for 4-6 times for complete dissolution. Do not shake with a vortex machine tool.
3. Filter this mixed blue solution with a filter using a 0.22-μm syringe filter attached in a 10-ml syringe.
4. Check the PH level of this solution to test of an appropriate validated method. When the PH level maintains in a constant range between 6.2 ~ 6.4 then load this AB solution to our own made specialized injection syringe prior to injection into the inguinal lymph node.

**2.3.4. DAPI:**

**Preparation of the DAPI Stock Solution:**

To make a 5 mg/mL DAPI stock solution (14.3 mM), dissolve the contents of one vial (10 mg) in 2 mL of deionized water or dimethylformamide (DMF).

Note: DAPI is not very soluble in phosphate-buffered saline (PBS).

For long-term storage, the stock solution can be aliquoted and stored at -< –20°C. For short- term storage, the solution can be kept at 2–6°C, protected from light. When handled properly, DAPI solutions are stable for at least six months.

**Preparation of the DAPI Working Solution:**

Dilute the DAPI stock solution to 300 nM in PBS. Add approximately 300 μl of this dilute DAPI staining solution.

**2.3.5. Phalloidin:**

Combine 10 μl of 6.6-μM Alexa Fluor 488 phalloidin and 300 μl of PBS. Use the sonicator to mix these well and store the solution at -20 °C. Avoid exposure to light.

**Movie caption:**

An experimentalist shook a lymph duct gently, and the blue threadlike structure should remain unbroken. The aggregates of dye would easily get broken. The PVS undulated in this process and remained intact as shown in the movie (Supplementary Information).