

Intravital imaging of embryonic and tumor neovasculature using viral nanoparticles

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Viral nanoparticles are a novel class of biomolecular agents that take advantage of the natural circulatory and targeting properties of viruses to allow the development of therapeutics, vaccines and imaging tools. We have developed a multivalent nanoparticle platform based on the cowpea mosaic virus (CPMV) that facilitates particle labeling at high density with fluorescent dyes and other functional groups. Compared with other technologies, CPMV-based viral nanoparticles are particularly suited for long-term intravital vascular imaging because of their biocompatibility and retention in the endothelium with minimal side effects. The stable, long-term labeling of the endothelium allows the identification of vasculature undergoing active remodeling in real time. In this study, we describe the synthesis, purification and fluorescent labeling of CPMV nanoparticles, along with their use for imaging of vascular structure and for intravital vascular mapping in developmental and tumor angiogenesis models. Dye-labeled viral nanoparticles can be synthesized and purified in a single day, and imaging studies can be conducted over hours, days or weeks, depending on the application.

INTRODUCTION

The visualization of vasculature in animal models is a fundamental tool in developmental biology and pathobiology, during which changes in vascular morphology can be highly dynamic and involve processes such as proliferation, migration, sprouting and remodeling (reviewed by Carmeliet *et al.*¹). Recent innovations in microscopy and imaging technologies are increasing the use of optical imaging for the study of vasculature, and the development of highly sensitive and specific contrast agents has enabled the real-time structural and functional intravital imaging of vascular beds. Plant-based viral nanoparticles represent a promising new generation of contrast agents because they combine the multivalent display of imaging moieties with a structurally well-defined and naturally biocompatible platform^{2–4}. Here we present protocols detailing the synthesis of fluorescent viral nanoparticles from the cowpea mosaic virus (CPMV) and describe their application for the imaging of mouse vasculature and human tumor vasculature in an avian embryo model. We also describe experiments for intravital vascular mapping, which allows the selective labeling and visualization of neovasculature that has formed within a prescribed time period in a live animal. These protocols are well suited to the analysis of dynamic processes related to blood vessel morphology and function, such as the characterization of germline mutations in genetically engineered models or the assessment of the impact of gain or loss of function of genes on angiogenesis in xenograft tumor models.

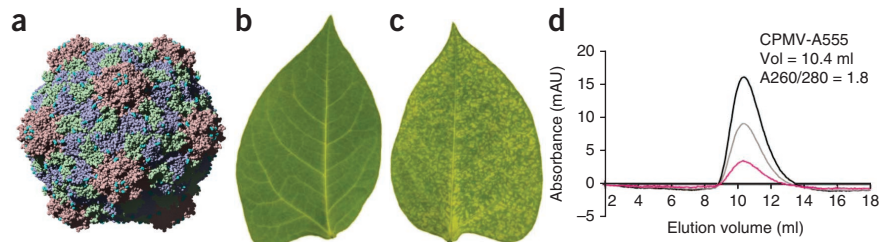
We recently established a viral nanoparticle platform based on CPMV to visualize tissues deep inside living organisms using high-resolution intravital imaging⁵. This approach allows the visualization of vasculature of living mouse and chick embryos to a depth of 500 μm for extended (> 72 h) periods of time⁵. CPMV comprises a 31-nm proteinaceous icosahedral capsid with 60 asymmetrical units⁶ (Fig. 1a). Each asymmetrical unit has five accessible lysine residues on the exterior surface that can be exploited for chemical

conjugation of functional moieties, for a total of 300 addressable sites per virion. We and others have described the attachment of a variety of functional moieties to CPMV capsids, including fluorescent dyes^{5,7,8}, quantum dots⁹, heavy metals^{7,10}, antibodies¹¹, peptides^{12,13}, proteins^{12,14} and polyethylene glycol^{5,15–18}. We have shown that CPMV viral nanoparticles labeled with 120 fluorescent dye molecules per virion exhibit no measurable quenching of fluorescence due to unfavorable dye-dye or dye-amino acid interactions⁵. We have also shown that CPMV-based viral nanoparticles decorated with multiple functional groups can be used for molecular targeting of receptors *in vivo*^{15,16}.

Cowpea mosaic virus nanoparticles injected intravenously are rapidly taken up by the vascular endothelium in vertebrates⁵. This behavior is mediated by the specific interaction of the CPMV capsid with the intermediate filament protein vimentin¹⁹. The uptake of CPMV nanoparticles does not affect vessel patency⁵, and the nanoparticles are retained within the endothelium without an appreciable signal loss or a change in localization over extended periods of time⁵. This unique endothelial uptake and retention behavior can be exploited to conduct long-term intravital imaging experiments or to persistently label the endothelium at specific time points and visualize changes in vascular structure over time. Uptake of CPMV nanoparticles is also seen in cells of the immune system¹⁹, allowing the visualization of inflammation²⁰. The decoration of CPMV nanoparticles with PEG polymers inhibits the binding of the viral capsid to a variety of cell types^{5,15,18}, resulting in a marked increase in plasma half-life¹⁰ and allowing the visualization of blood flow in living organisms⁵. For subsequent pathological tissue analysis, CPMV nanoparticles retain their fluorescence and localization after fixation in paraformaldehyde, formaldehyde or acetone⁵. CPMV nanoparticles can be labeled to high density using straightforward chemistry with commercially available dyes, and virus conjugates are stable for up to 1 year when stored at 4 °C. These favorable characteristics make CPMV an ideal *in vivo* imaging agent^{7,8,21–23}.



Figure 1 | The cowpea mosaic virus (CPMV) is a multivalent platform for fluorescent dye conjugation. **(a)** The space-filling model of CPMV ultrastructure assembled from coordinates obtained from [http://viperdb.scripps.edu/\(ref.50\)](http://viperdb.scripps.edu/(ref.50)). **(b)** Primary leaf of black-eyed pea plant. **(c)** Mosaic virus infection symptoms on secondary leaf, 7–10 d after inoculation with CPMV. **(d)** Chromatogram of Alexa 555-labeled CPMV nanoparticles. Red, gray and black lines represent absorbance at 555 nm, 280 nm and 260 nm, respectively.



Several contrast agents have been described for the imaging of the vascular endothelium, including microspheres or nanospheres²⁴, metal nanoparticles^{25–27}, liposomes^{28,29}, dextrans³⁰, lectins³¹, antibodies³² and quantum dots^{33,34}. Each of these tools has advantages and disadvantages that must be considered. The majority of these contrast agents are restricted to the blood pool and do not explicitly label the endothelium, resulting in a rapid decline in vascular signal within 4 h after intravenous administration⁵. Fluorescent lectins provide excellent short-term visualization of the endothelium because of their specific binding to glycoproteins on the luminal surface of endothelial cells, yet they show a similar rapid decline in signal intensity over time⁵. Although fluorescent CD31-directed antibodies have been used with success for intravital imaging of the vascular endothelium beyond 12 h (refs. 35,36), they can block leukocyte diapedesis at sites of inflammation³⁷. Furthermore, the systemic persistence and elimination of synthetic nanomaterials should also be considered for long-term experiments. For example, the biocompatibility of inorganic materials such as quantum dots can be improved by coating with biodegradable materials, yet the heavy metal core is, by nature, nonbiodegradable, which can result in cytotoxic effects^{38–42}. Other nanomaterials, such as gold, may not have significant toxicity *in vivo*⁴³.

The advantages of using CPMV nanoparticles for intravital vascular imaging are numerous. They can be labeled with up to 200 fluorescent dye molecules without measurable quenching¹⁵, resulting in exceptionally bright particles that effectively increase the maximum depth of imaging. Because they persistently label the endothelium without toxicity, a single injection of CPMV nanoparticles can be used to image the endothelium continuously for 3 d or more. Furthermore, their rapid uptake and entrapment by endothelial cells allows the precise mapping of vasculature at distinct time points. Nevertheless, the use of CPMV nanoparticles has several potential limitations. Because CPMV nanoparticles are internalized by endothelial cells *in vivo* and localize to perinuclear endosomes, the luminal surface of the endothelium is not precisely delineated. The plasma half-life of CPMV in mice is ~7 min, making the measurement of blood flow difficult. Because PEGylated CPMV has a much longer plasma half-life, it is better suited for these analyses. Furthermore, we observe a marked difference in the labeling efficiency of arterial and venous vasculature, in which

venous blood vessels accumulate approximately three times the signal of arterial vessels. This must be incorporated into the experimental plan if arterial vasculature is the focus of detailed studies. Finally, production of CPMV in its host plant (*Vigna unguiculata*, commonly known as black-eyed pea) results in infectious particles. This may be undesirable for certain applications, although it must be noted that these viral nanoparticles will neither infect nor replicate in animals. Although noninfectious particles can be isolated after purification from their RNA⁴⁴ or induced by UV inactivation⁴⁵, intact noninfectious CPMV capsids can now be produced in insect cells⁴⁶.

In this protocol, we describe the propagation and purification of CPMV nanoparticles from black-eyed pea plants. We then explain the steps required to conjugate fluorescent dyes to viral nanoparticles, purify them and quantify the level of dye conjugation. We go on to describe the steps required to perform intravital imaging of vasculature in mouse embryos and human tumors in a shell-less avian embryo model, followed by the procedures for implanting xenograft tumors onto the chorioallantoic membrane (CAM) of the avian embryo for the temporal analysis of tumor angiogenesis and intravital vascular mapping of tumor vasculature. These protocols allow the production of milligram quantities of dye-labeled CPMV nanoparticles within 3–4 weeks of primary leaf inoculation. Typical intravital imaging experiments require 5 μg of CPMV nanoparticles per animal; thus, each preparation will provide sufficient material for hundreds of imaging experiments. Although the virus propagation, preparation and purification methods described herein are optimized for CPMV nanoparticles, the protocols can be generalized to other plant-based viruses with minimal modification. Furthermore, the novel imaging methodologies used in this protocol can be applied to other nano-sized contrast agents that are labeled with fluorescent dyes. Fluorescent viral nanoparticles are not limited to the visualization of blood vessels, and we have successfully labeled and visualized lymphatic vessels in live animals. The intravital imaging procedures described here use embryonic models, mainly because of tissue characteristics that are favorable for deep-tissue imaging. Although viral nanoparticles behave similarly in adult tissues, it would be recommended to use two-photon imaging to adequately visualize structures deeper than 200 μm.

MATERIALS

REAGENTS

- Mouse embryos can be chosen depending on the design of the experiment (CD1 mouse embryos were used in this study)
- Fertilized white leghorn eggs ▲ **CRITICAL** All experiments were conducted in accordance with the regulations and guidelines of the Institutional Animal Care and Use Committee at the University of California,

San Diego, and the Animal Care and Use at the University of Western Ontario.

- Alexa Fluor 750 carboxylic acid (succinimidyl ester, Invitrogen, cat. no. A20011)
- Alexa Fluor 647 carboxylic acid (succinimidyl ester, Invitrogen, cat. no. A20006)

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- Alexa Fluor 555 carboxylic acid (succinimidyl ester, Invitrogen, cat. no. A20009)
- Alexa Fluor 488 carboxylic acid (succinimidyl ester; Invitrogen, cat. no. A30006)
- Methyl-PEO₄-NHS ester (Pierce, cat. no. PI22341)
- PEG (8,000 MW for precipitation, BDH, cat. no. B80016)
- Carborundum (Fisher, cat. no. C192-500)
- CPMV virions **! CAUTION** Note that in the United States, the laboratory use of infectious plant viruses is regulated by the United States Department of Agriculture (USDA).
- Distilled water (dH₂O)
- Dimethyl sulfoxide (DMSO; Sigma, cat. no. D8418)
- Dulbecco's PBS (pH 7.2–7.4) (Invitrogen, cat. no. 14190250)
- DMEM/F12 media (Invitrogen, cat. no. 11330-057)
- Heat-inactivated fetal bovine serum (Invitrogen, cat. no. 12483020)
- Penicillin-streptomycin liquid (100 ml, pen-strep; Invitrogen, cat. no. 15140122)
- Trypsin-EDTA (2.5×, Invitrogen, cat. no. 25300054)
- HEPES buffer (1 M, pH 7.2, Bioshop, cat. no. HEP003.100)
- Mineral oil (Sigma, cat. no. M8410)
- Paraformaldehyde in PBS (4% (vol/vol), pH 7.2–7.4) **! CAUTION** Do not breathe fumes; carry out the experiments under a fume hood whenever possible.
- Ethanol (70% (vol/vol))
- Sucrose (Sigma, cat. no. S0389)
- Vacuum grease (VWR, cat. no. 59344-055)
- Dibasic monohydrogen phosphate (K₂HPO₄, Sigma-Aldrich, cat. no. 379980)
- Monobasic dihydrogen phosphate (KH₂PO₄, Sigma-Aldrich, cat. no. P5655)
- 1-Butanol (Sigma, cat. no. B7906)
- Sodium chloride (Sigma, cat. no. S7653)
- *V. unguiculata* seeds (California black-eye no. 5; Burpee, cat. no. 51771A)

EQUIPMENT

- Avanti JE centrifuge (Beckman)
- Beckman Coulter DU 800 spectrophotometer (Beckman)
- Superose 6 size-exclusion column
- ÄKTA explorer
- Dissecting microscope
- Fiber-optic microscope illuminator (150W, Amscope, model HL250-AY)
- Epifluorescence wide-field microscope (Quorum; Zeiss Axio Examiner, Zeiss)
- Spinning disk confocal fluorescence microscope (Quorum; Yokogawa CSU 10, Yokogawa)
- Temperature enclosure unit for microscope (Precision Plastics)
- Roller culture unit
- Sportsman hatcher (Berry Hill, cat. no. 1550HA)
- Sportsman incubator (Berry Hill, cat. no. 1502EA)
- Rubbermaid rubber container with lid (Guillevin, cat. no. RH3-228-00-BLU) and holes drilled into sides
- Vertical pipette puller (David Kopf Instruments, model 720)
- Sodium borosilicate glass capillary tubes (OD, 1.0 mm; ID 0.58 mm; 10-cm length; Sutter Instrument, cat. no. BF100-58-10)
- Grade-1 Whatman filter paper (VWR, cat. no. 28450-070)
- Circular glass coverslips no. 1 (18 mm, VWR, cat. no. 16004-300)
- Fine-point forceps (VWR, cat. no. 25607-856)
- Paper hole puncher

- Hemocytometer (Hausser Scientific, VWR, cat. no. 15170-090)
- Pyrex Petri dish (100 × 20, VWR, cat. no. 89000-306)
- Two-chamber/coverglass wells (Nunc Lab-Tek, cat. no. 155380)
- Tygon R-3603 laboratory tubing, 50 ft (1/32-inch inner diameter, 3/32-inch outer diameter, 1/32-inch wall thickness; VWR, cat. no. 63009-983)
- Syringes for injections (1 ml, box of 100; BD, cat. no. 309602)
- Syringes (20 ml, box of 100; BD, cat. no. 521906)
- Hypodermic needles for injections (18- and 27.5-gauge needles, box of 100; BD, cat. nos. 305195 and 305196)
- Intellitemp heat mat (19.7 × 11.8 inches, 30 W, Big Apple Herpetological)
- Dremel tool
- Dremel cutoff wheels no. 36 (Dremel, cat. no. 409))
- Polystyrene weigh boats (VWR, cat. no. 12577-01)
- Square Petri dishes (Simport, VWR, cat. no. 25378-115)
- MM-33 micromanipulator (Fine Science Tools)
- Microadjustable syringe pump (Braintree Scientific)
- Vacuum aspiration unit with 0.5-inch tubing
- Glass Pasteur pipettes (VWR, cat. no. 14673-010)
- Embryo imaging unit (Quorum Technologies)
- Indoor growth lights (for example, SunLite, Gardener's Supply at <http://www.gardeners.com>) Disposable UV-transparent disposable cuvettes (box of 100; Sarstedt, cat. no. 67.758)

REAGENT SETUP

Mouse embryo dissecting medium Mouse embryo dissecting medium is composed of 100 ml of DMEM/F12 medium supplemented with 9 ml of heat-inactivated fetal bovine serum and 1 ml of pen-strep solution. This can be prepared in advance and stored at 4 °C for 1 month.

Mouse embryo culturing medium Mouse embryo culturing medium is composed of 50 ml of DMEM/F12, 50 ml of heat-inactivated fetal bovine serum, 20 µl of pen-strep solution and 20 µl of 1 M HEPES buffer solution. The medium should be equilibrated at 37 °C in an incubator for 1 h before use. Prepare in advance and store at 4 °C for 1 month.

Potassium phosphate buffer (0.1 M, pH 7.0) Dissolve 10.7 g of K₂HPO₄ and 5.24 g of KH₂PO₄ in 100 ml of dH₂O. Bring to a volume of 1 liter with water. This combination of salts should result in a solution pH of 7.0. Prepare in advance and store at 4 °C for 1 month.

Sodium chloride solution (NaCl, 2 M) Dissolve 11.7 g of sodium chloride in 100 ml of dH₂O. Prepare in advance and store at 4 °C for 1 month.

EQUIPMENT SETUP

- To prepare glass needles for injection, prepare microinjection needles drawn from borosilicate glass capillary tubes using a pipette puller. Prepare needles that are as long and slowly tapered as possible by adjusting the heat and solenoid settings on the puller. Settings on the 720 Kopf model are generally 16.3 (heater) and 2.3 (solenoid). These needles can be prepared ahead of time and stored in a sterile dish.
- For confocal microscopy, we use a Zeiss Examiner Z1 upright microscope fitted with a Yokogawa spinning disk, Hamamatsu Imagem 9100-12 EM-CCD camera and diode lasers (405, 491, 561, 647 nm). Although an inverted microscope can be used for mouse embryo imaging experiments, avian embryos must be visualized using an upright microscope.
- For wide-field microscopy, we use a Zeiss Examiner Z1 upright microscope fitted with a Hamamatsu 9100-02 EM-CCD camera.

PROCEDURE

Propagation of CPMV in plants ● TIMING 3–4 weeks

1| Seed *V. unguiculata* seeds in standard potting soil. Growth conditions require lighting for 16 h a day (08:00–12:00 hours), at 25 °C and 60% humidity. Plants may be grown in an outdoor greenhouse or indoors using growth lights. The plants must be watered at ~48-h intervals to keep the soil moist but not soggy.

2| Approximately 10–12 d after sowing and when the primary leaves (**Fig. 1b**) have appeared, infect leaves by mechanical inoculation with CPMV. Coat leaves with a fine dusting of carborundum powder. Pipette 5 µg of purified CPMV virions diluted in 50 µl of 0.1 M potassium phosphate buffer (pH 7.0) onto the surface of each leaf, and then rub the leaves gently to wound the leaf surface and allow virus infection. Wash off the carborundum with tap water ~10 min after inoculating the plants.

3| Mosaic virus infection symptoms will appear on primary leaves ~7–10 d after inoculation. Symptoms on trifoliolate leaves will be detectable after a further 7–10 d (**Fig. 1c**). Harvest all leaves when trifoliolates show symptoms.

4| Collect leaves in a reclosable plastic bag and store at –20 or –80 °C.

■ **PAUSE POINT** Leaves can be frozen indefinitely.

Purification of CPMV from infected leaves ● **TIMING 10–12 h**

5| Add 100 g of frozen leaves to a standard blender. Add two volumes of cold 0.1 M potassium phosphate buffer (pH 7.0) and homogenize the leaf material until it is smooth.

6| Filter the homogenized plant material into a flask through a sterile cheesecloth.

▲ **CRITICAL STEP** Squares of cheesecloth should be sterilized before use by wrapping in aluminum foil and autoclaving using a dry cycle.

7| Spin down crude plant material by centrifugation at 11,113g at 4 °C for 20 min using a JLA 16.25 rotor (Avanti JE centrifuge, Beckman) and transfer the supernatant into a flask.

8| Extract plant material by adding 0.7 volumes of a 1:1 (vol/vol) mixture of chloroform/1-butanol. Stir the mixture at 4 °C for 30–60 min.

9| Centrifuge at 3,629g in a JLA 16.250 rotor (Avanti JE centrifuge, Beckman) for 10 min at 4 °C. Collect the upper aqueous phase (containing CPMV particles) into a flask.

10| To PEG-precipitate the particles, add 0.1 volume of 2 M NaCl and 80% (wt/vol) PEG (molecular weight 8,000) and stir for 60 min (or longer) at 4 °C.

11| Centrifuge at 15,000g in a JLA 16.250 rotor for 15 min at 4 °C. Discard the supernatant and resuspend the pellet in 10–20 ml of 0.1 M potassium phosphate buffer (pH 7.0) by pipetting up and down.

12| Centrifuge at 9,047g using a JA-17 rotor for 15 min at 4 °C. Collect the supernatant and discard the pellet.

13| Pellet CPMV particles by ultracentrifugation at 160,326g using a Beckman Type 50.2 Ti rotor for 3 h at 4 °C.

14| Resuspend the pellet in 2–5 ml of 0.1 M potassium phosphate buffer (pH 7.0).

15| Purify the CPMV particles by layering them over a 10–40% sucrose gradient in 0.1 M potassium phosphate buffer (pH 7.0) using a Beckman SW32 rotor at 101,156g for 3 h. Refer to reference 47 for a detailed description of how to set up a sucrose density gradient for purification of virus particles.

16| Using a 20-cc syringe and 18-gauge, 1 ½-inch needle, extract CPMV bands under visible light by puncturing the tube slightly from the side under the protein band and extracting the band. The band should appear white.

17| Pellet CPMV particles again by ultracentrifugation at 160,326g using a Beckman 50.2 fixed-angle rotor for 3 h at 4 °C.

18| Resuspend the pellet in 2–5 ml of 0.1 M potassium phosphate buffer (pH 7.0).

19| Determine the concentration of the particles by ultraviolet-visible light spectroscopy. In detail, pipette 1 ml of virus preparation into a ultraviolet-visible spectroscopy cuvette. For blank measurement, add 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) to the cuvette and measure as ‘blank’ on a Beckman Coulter DU 800 spectrophotometer. Thereafter, determine the absorbance of the virus preparation at 260 nm and use the formula and extinction coefficient data in Step 23A(i) to quantify the virus concentration.

Conjugation of CPMV with fluorescent dyes ● **TIMING At least 8 h (optional overnight incubation)**

20| Dissolve Alexa Fluor 488/555/647/750 (carboxylic acid, succinimidyl ester) dyes in dry DMSO and add to the pelleted CPMV particles in 0.1 M potassium phosphate buffer (pH 7.0) (from Step 18) at 2–3 mg ml⁻¹ using a molar excess of 3,000:1 dye molecules per CPMV. Adjust the final DMSO concentration to 20% (vol/vol).

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21| Incubate the reaction mixture on a rotating shaker for 2 h at room temperature (22 °C) (or overnight at room temperature to ensure maximum labeling).

22| Purify samples by ultracentrifugation in a 10–40% sucrose gradient, followed by ultracentrifugation, as described above (see Steps 14–18).

Characterization of CPMV-dye conjugates ● **TIMING** Step 23(A), 0.5–1 h; Step 23(B), 2 h

23| CPMV-dye conjugates can be further characterized using either ultraviolet-visible spectroscopy (option A) or size-exclusion chromatography (SEC; option B).

(A) Determination of concentration and labeling efficiency using ultraviolet-visible spectroscopy

(i) Determine the concentration of CPMV particles and the concentration of each fluorescent dye using the standard equation: absorbance (A) = extinction coefficient ϵ × path length (in cm) × concentration by ultraviolet-visible spectroscopy using a Beckman Coulter DU 800 spectrophotometer.

▲ **CRITICAL STEP** The extinction coefficient of CPMV at 260 nm is $\epsilon = 8.1 \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml}$. The molecular weight of CPMV in moles can be calculated using its molecular weight, which is $5.6 \times 10^6 \text{ g mol}^{-1}$. Concentration is calculated with the following equation: concentration in g l^{-1} /molecular weight in g mol^{-1} = concentration in mol l^{-1} . The absorbance and emission maxima and the extinction coefficients of the Alexa Fluor dyes we use are as follows:

Alexa Fluor 750: absorbance 749 nm, emission 775 nm, $\epsilon = 290,000 \text{ cm}^{-1} \text{ mol}^{-1}$

Alexa Fluor 647: absorbance 650 nm, emission 665 nm, $\epsilon = 270,000 \text{ cm}^{-1} \text{ mol}^{-1}$

Alexa Fluor 555: absorbance 555 nm, emission 565 nm, $\epsilon = 155,000 \text{ cm}^{-1} \text{ mol}^{-1}$

Alexa Fluor 488: absorbance 496 nm, emission 519 nm, $\epsilon = 73,000 \text{ cm}^{-1} \text{ mol}^{-1}$

(ii) Determine the number of dye molecules per particle as follows:

Number of dye molecules per CPMV = concentration of dyes in mol ml^{-1} /concentration of CPMV in mol ml^{-1} .

(B) SEC of native and dye-conjugated CPMV particles

(i) To verify that dye molecules are covalently attached and CPMV particles remain structurally sound, analyze particles by SEC using a Superose 6 size-exclusion column and the ÄKTA Explorer. Load 100 μg of particles into 500 μl of 0.1 M potassium phosphate buffer (pH 7.0) and analyze at a flow rate of 0.5 ml min^{-1} . Set detectors to 260 nm (detection of the RNA component) and 280 nm (detection of the protein component). Set a third wavelength detector to the respective absorbance maximum of the specific dye attached.

(ii) Compare the elution profiles of native and chemically modified particles to determine whether CPMV particles are intact. Native CPMV and dye-conjugated CPMV particles elute at the same volume as a single peak. The ratio of absorbance measured at 260–280 nm for intact particles is 1.8. Chromatograms of dye-labeled particles will show a third peak at the dye-specific wavelength. Coelution of the peak with peaks detected at 260 nm and 280 nm indicate that the dyes are covalently attached to the particles (**Fig. 1d**).

24| To use dye-conjugated CPMV particles to analyze mouse embryo vasculature, use option A for the preparation and *ex vivo* culture of mouse embryos. To analyze human tumor angiogenesis in avian embryos, use option B for the preparation and *ex ovo* culture of avian embryos and the inoculation of tumor cells into the avian embryo CAM (**Fig. 2**).

(A) Preparation and *ex vivo* culture of mouse embryos ● **TIMING** 1 h (plus a total of 8–15 d to obtain mouse embryos)

(i) Combine male and female breeder mice in a single cage and designate female mice with a vaginal plug as 0.5 d post coitum (dpc). For more information regarding the assessment of vaginal plugs, see <http://jaxmice.jax.org/jaxnotes/archive/501d.html>.

(ii) At 8–15 dpc, kill female mice with CO_2 and subsequent cervical dislocation.

(iii) Using scissors, perform lengthwise incision along the median of the abdomen. Thereafter, make two large flaps so that the lower abdomen is exposed. Extract uterine horns and immerse them in dissecting medium warmed to 37 °C.

(iv) Gently tease apart individual embryos. The yolk sac and placenta should be kept attached to each embryo to preserve embryonic vasculature and blood flow.

(v) Transfer embryos to chamber slides containing 2 ml of culturing medium. Ensure that there are no more than three embryos per chamber well.

(B) Preparation and *ex ovo* culture of avian embryos and inoculation of tumor cells into avian embryo CAM

● **TIMING** 1–3 h (plus a total of 8–15 d to culture avian embryos)

(i) On receipt of fertilized white leghorn eggs (day 0), incubate for 4 d at 38 °C at 60% humidity in a hatcher with rotation.

(ii) On day 4, assemble a Dremel drilling tool with a circular wheel (no. 36 cutoff, diameter of 15/16 inch) onto a rod attached to a suitable base, and fix at chest level using a three-prong clamp.

(iii) Spray the Dremel drilling tool with 70% ethanol to sterilize it.

▲ **CRITICAL STEP** If the Dremel tool is not properly sterilized, many of the shell-less embryos will become contaminated after the procedure.

- (iv) To deshell fertilized eggs, hold both ends of the egg with both hands and lightly coat the full ‘waist’ of the egg shell with 70% ethanol in a weigh boat.
 - ▲ **CRITICAL STEP** Keep the egg lengthwise in the same position for at least 10 s so that the embryo rotates to the top.
- (v) With the Dremel drilling tool on medium to high setting, make four shallow cuts equidistant along the circumference of the shell (**Fig. 2a**).
 - ▲ **CRITICAL STEP** Cuts should be 1–1.5 inches long and equidistant from each other. This will make separating the eggshell much easier.
- (vi) On a new weigh boat (previously sprayed with 70% ethanol and dried), lightly rock the egg back and forth while applying pressure along the top and sides of the axis of cuts so that the egg shell splits along the axis. Next, pull the two halves up and apart, gently releasing the embryo into the weighing dish (**Fig. 2b**).
 - ▲ **CRITICAL STEP** A visible heartbeat indicates a healthy embryo. If it is not present, the embryo should be discarded.
 - ▲ **CRITICAL STEP** For optimal results and viability, fertilized eggs should be removed from their shells on day 4.
- (vii) Place a sterile square plastic cover over the weigh boat and transfer to a rubber container with holes drilled into the sides; the container should be filled with about a half-inch of distilled water. Place six square Petri dishes at the bottom, top-side up, to elevate the weigh boats containing the embryos above the water. Embryos can be stacked three high; the container can hold approximately 18 embryos. Incubate the container in a clean humidified incubator at 38 °C at 60% humidity. Use embryos at any point after day 8 of incubation.
 - ? **TROUBLESHOOTING**
- (viii) Before inoculation of tumor cells into the avian embryo CAM, culture a cancer cell line of interest with no antibiotic or selective medium to 80% confluency. Refer to ref. 48 for full details on techniques on how to properly culture all types of cancer cell lines.
 - ▲ **CRITICAL STEP** Allowing cells to grow to greater than 80% confluency will decrease tumor uptake or extravasation efficiency.
- (ix) To trypsinize cells, wash them twice with 1× PBS (pH 7.4). Aspirate the remaining PBS, then add 0.5% trypsin-EDTA (for example, add 2 ml to a T75 flask, 3 ml to a T175 flask or 3 ml to a 150-mm culture dish) and incubate at 37 °C for 2–5 min until all cells detach.
- (x) Add 7–8 ml of serum-containing medium to neutralize the trypsin and transfer cell suspension to a 15-ml Falcon tube.
- (xi) Centrifuge at room temperature at 200g for 5 min.
- (xii) Aspirate the supernatant and resuspend in 10 ml of PBS, then repeat Step 24B(xi).
- (xiii) Pour out the supernatant, resuspend cells in 1,000 μl of PBS and transfer to a 1.5-ml Eppendorf tube.
- (xiv) Take 10 μl of suspension and dilute into 490 μl of PBS. Count the number of cells in the diluted suspension using a hemocytometer.
- (xv) Concentrate cells to 5 × 10⁷ cells per ml. Use only 1× PBS to dilute and/or resuspend cell concentrates.
- (xvi) Count cells and resuspend to a final concentration of 1 × 10⁷ cells per ml.
 - ▲ **CRITICAL STEP** Proceed to implantation immediately after preparation.
- (xvii) Prepare microinjector by fitting an 18-gauge needle onto a 1-ml syringe. Cut a 5–6-inch piece of Tygon tubing and carefully insert the bevel of the needle into the bore of the tubing. Slowly slide the tubing all the way onto the needle. There should be about 4–5 inches of tubing extending from the tip of the needle.

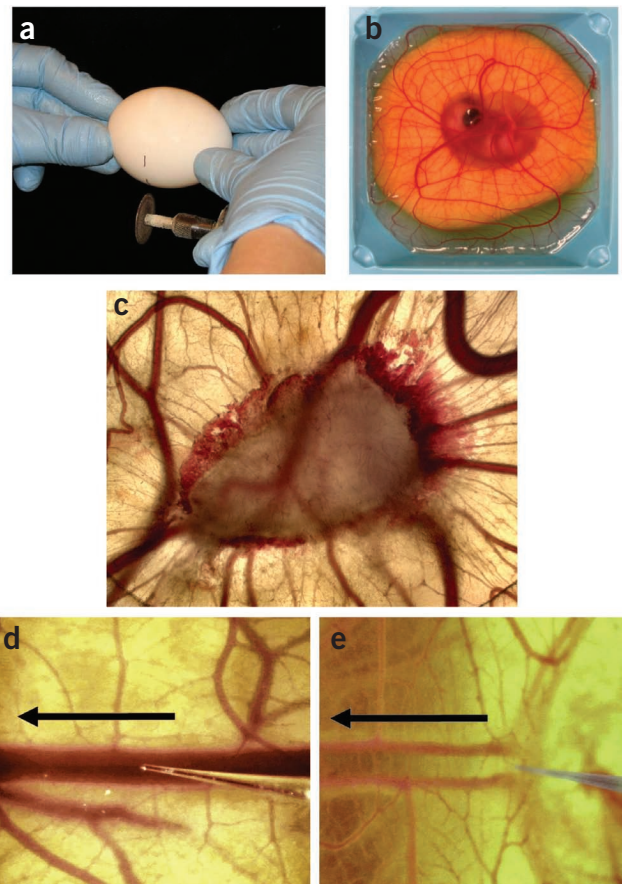
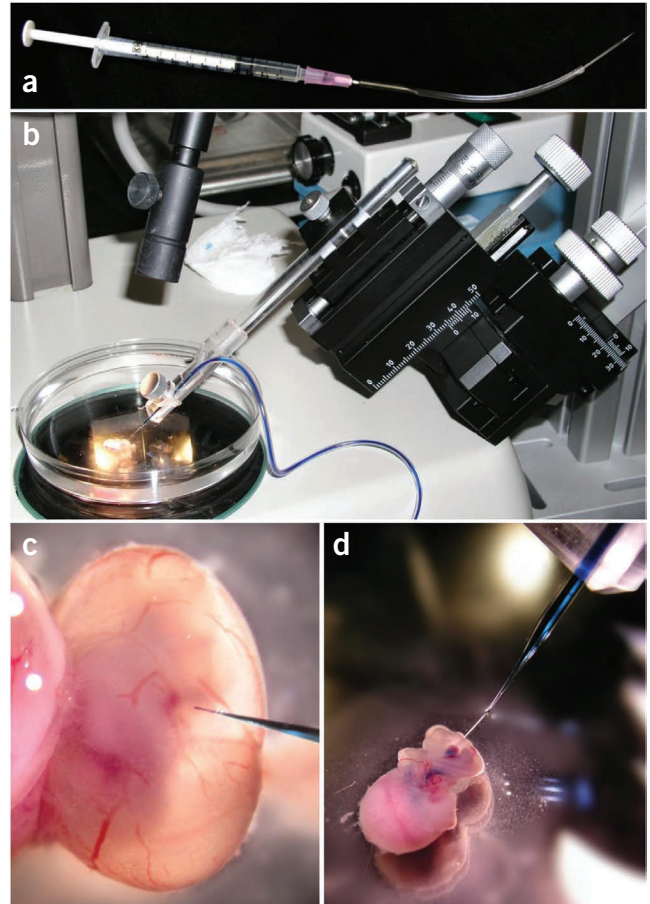


Figure 2 | Preparation of shell-less avian embryos. (a) Removal of shell from avian embryo using a Dremel tool. (b) Day 11 avian embryo showing the highly vascularized CAM on the top surface. (c) Bright-field imaging of topical tumor implanted onto the surface of CAM. (d) Microinjection needle tip approaching the CAM vein. Black arrow indicates blood flow direction. (e) Microinjection needle inserted into vessel lumen; dye-labeled CPMV nanoparticles are being injected and visualized entering the blood flow.

PROTOCOL

Figure 3 | Microinjection of CPMV nanoparticles into mouse embryos. (a) Assembled microinjection syringe. (b) Setup of micromanipulator, microinjector and Petri dish with a single mouse embryo in prewarmed dissection medium. (c) Mouse embryo (day 12.5) with an intact yolk sac. Microinjection needle approaching a yolk sac vein to inject dye-labeled viral nanoparticles. (d) Intracardiac injection of fluorophore-labeled CPMV particles into mouse embryo with yolk sac removed.



(xviii) Fill the syringe with cell suspension and add the glass needle, removing air bubbles.

▲ **CRITICAL STEP** If the cells clump within the syringe or clog the needle, cut off the glass needle, remix the cell suspension and insert a new needle.

? TROUBLESHOOTING

(xix) Using a dissection scope with an illuminator, inject day 8 to day 10 embryos (from Step 24B(vii)) with 10,000–100,000 cancer cells as a bolus within the CAM. Slowly inject cells; if the needle is in the correct position, the cells will form a visible bolus within the CAM. Avoid dripping cells onto the CAM surface. If this occurs, dab with a clean Kimwipe or cotton-tipped applicator.

(xx) Return the embryos to the humidified incubator and allow the tumor to grow for the desired time (up to 7 d).

? TROUBLESHOOTING

Injection of fluorescently labeled viral nanoparticles

● TIMING 1 h

25 | Assemble the injection apparatus by fitting an 18-gauge needle onto a 1-ml syringe. Cut a piece of Tygon tubing (3–4 inches long) and carefully insert the bevel of the needle into the lumen of the tubing. Slowly slide the tubing all the way onto the needle. There should be about 2–3 inches of free tubing.

26 | Draw the desired volume of CPMV nanoparticles through the tubing into the syringe (>200 μ l). Remove dead space and any air bubbles.

27 | Prepare a microinjection glass needle that can be drawn from sodium borosilicate glass capillary tubes and a needle puller (this step can be performed in advance).

28 | Position the blunt end of the glass needle at the end of the tubing and push the syringe to allow the liquid to meet the glass tip. It may be necessary to break the needle back with fine forceps so that the particles can be easily injected. A fully assembled injection apparatus is shown in **Figure 3a**.

▲ **CRITICAL STEP** Prepare needles that are as long and tapered as possible. If they are too blunt, the needle will not pierce through the ectoderm and will fail to penetrate into the vessel of interest; if too sharp, the tip will collapse when penetrating the tissue.

29 | To allow intravital imaging, mouse embryos (from Step 24A(v)) are injected with fluorescent CPMV nanoparticles through the yolk sac vein (using option A) or by intracardiac injection (option B); alternatively, option C can be used to inject fluorescent viral nanoparticles into tumor cells inoculated into the avian embryo CAM (from Step 24B(xx)).

(A) Injection into the yolk sac vein of mouse embryo ● TIMING 30 min

(i) Place a single embryo into a Petri dish containing prewarmed dissecting medium.

(ii) Using a dissecting microscope and micromanipulator (**Fig. 3b**), slowly penetrate the tip of the needle into the yolk sac vein distal to the desired site to be visualized (**Fig. 3c**).

(iii) Lightly tap the barrel of the injection apparatus and look for flow of CPMV particles into the vessel. If flow is present and there is no resistance when force is applied to the syringe barrel, gently expel \sim 50 μ l of diluted CPMV particle solution (100 μ g ml⁻¹ of CPMV particles in PBS), which is enough to label the entire vasculature of a single embryo.

? TROUBLESHOOTING

- (iv) If there is no flow or if there is resistance when force is applied to the syringe barrel, slowly retract the tip away from the yolk sac vein and try another yolk sac vein.

▲ **CRITICAL STEP** The needle must be very sharp, with a very narrow tip bore. If the needle is blunt, it will not penetrate the yolk sac vein.

? **TROUBLESHOOTING**

- (v) Alternatively, perform manual injection of CPMV particles (without the use of the micromanipulator). Ensure maximum stability of the needle during injection of CPMV particles into the vessel lumen and restrict movement of the injection apparatus to the force applied to the syringe barrel.
- (vi) Incubate for 5–10 min before imaging.

(B) Intracardiac injection of mouse embryo ● TIMING 30 min

- (i) Place a single embryo into a Petri dish containing prewarmed dissecting medium.
- (ii) Using forceps and scissors, gently remove the yolk sac from the embryo. Take great care and do not puncture the embryo.
- (iii) Transfer the embryo to another Petri dish with fresh prewarmed dissecting medium.
- (iv) Set up the injection apparatus as described in Step 24B(xvii), and set up the dissecting scope and micromanipulator as shown in **Figure 3b**. Ensure that there are no air bubbles in the injection apparatus.
- (v) Using the micromanipulator, approach the heart of the embryo at a 30–45° angle. If necessary, use forceps to hold the embryo in place. Alternatively, position the embryo against the wall of the Petri dish. Penetrate the needle tip into the heart and lightly tap the barrel of the injection apparatus and look for flow of CPMV particle solution into the heart (**Fig. 3d**).

? **TROUBLESHOOTING**

- (vi) If flow is present, gently expel ~50 µl of diluted CPMV, which is enough to label the entire vasculature of a single embryo (100 µg ml⁻¹ of CPMV in PBS).
- (vii) Performing multiple intracardiac injection attempts will substantially affect the viability of the animal. Therefore, in the event of intracardiac injection failure, discard embryo and move on to another embryo.
- (viii) Incubate for 5–10 min before imaging.

(C) Injection of labeled viral nanoparticles into tumor cells inoculated into the avian embryo CAM for tumor vascular mapping ● TIMING 0.5–1 h

- (i) Tumor vascular mapping is performed by injecting CPMV nanoparticles conjugated to distinct fluorescent labels at successive time points. In a typical experiment, CPMV nanoparticles conjugated to one fluorophore are injected intravenously at an initial time point. After a prescribed period of time (for example, 24 h), CPMV nanoparticles conjugated with a different fluorophore are injected intravenously. Microinject CPMV nanoparticles into a CAM vein distal to the desired site to be visualized (**Fig. 2d**). Successful cannulation of the CAM vein is evident by the clearing of blood in the path of the injection flow (**Fig. 2e**). A volume of 50 µl of diluted CPMV particle solution (100 µg ml⁻¹ of CPMV particles in PBS) is sufficient to label the entire vasculature of a single embryo.

? **TROUBLESHOOTING**

- (ii) At the second time point, microinject CPMV particles labeled with a different fluorophore into the CAM and visualize them under a fluorescent microscope. Incubate for 5–10 min before imaging. Additional time points may be incorporated into the procedure by using CPMV nanoparticles labeled with distinct fluorescent dyes.

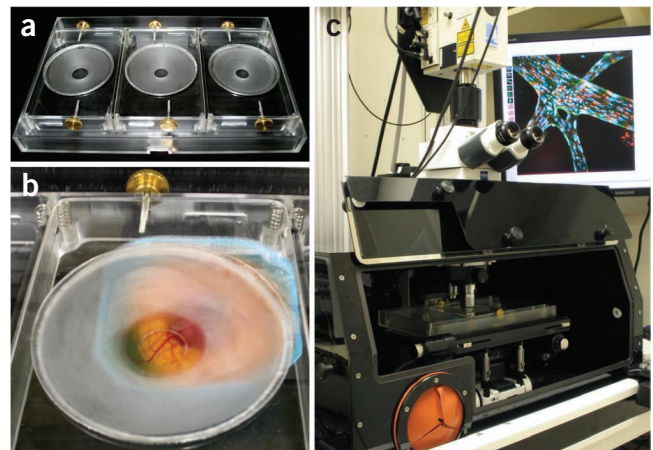
Intravital imaging ● TIMING Hours to days, depending on the experimental plan

30| Apply a thin layer of vacuum grease around the circumference of the imaging port on the underside of the lid of the embryo imaging unit (**Fig. 4a,b**), and then fit an 18-mm glass coverslip onto it. Wipe off excess vacuum grease.

31| Position the embryo so that the coverslip can be lowered directly onto the area of interest. Slowly lower the lid of the embryo imaging unit until the coverslip just makes contact with the embryo, without applying force to the tissue surface, and screw the lid in place (**Fig. 4b**).

▲ **CRITICAL STEP** Ensure that the lid is completely level using a small bubble level. This will minimize focal plane artifacts during imaging.

Figure 4 | Embryo imaging unit for intravital imaging of avian and mouse embryos. (a) Microscope-mounted incubation chamber for simultaneous intravital imaging of three avian embryos. (b) Avian embryo mounted in imaging chamber with direct contact between coverslip and CAM. (c) Microscope-mounted incubation chamber is placed on the stage of an upright confocal microscope contained within a temperature-regulated enclosure set to 37 °C.



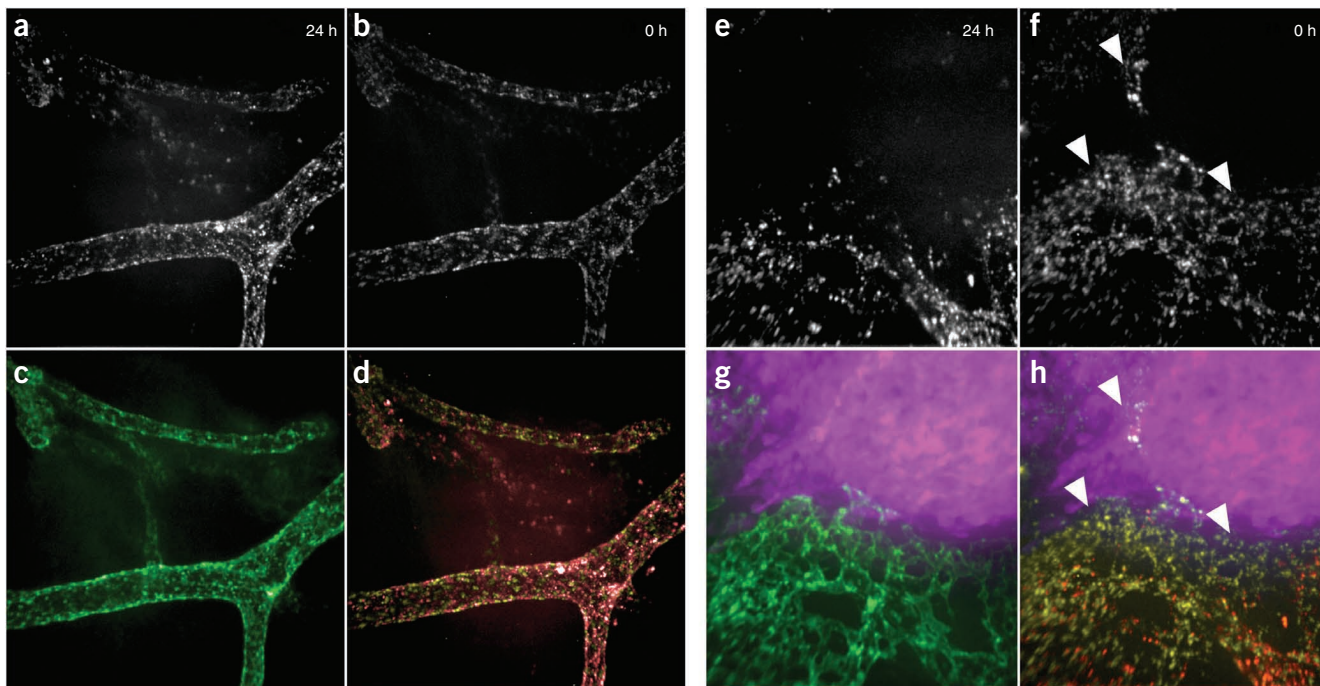


Figure 5 | Intravital vascular mapping of human tumors in the CAM. (a–d) Mapping of quiescent vasculature: (a) visualization of vasculature using CPMV-A647 24 h after injection; (b) visualization of vasculature using CPMV-A750 immediately after injection; (c) visualization of endothelium labeled with fluorescein lectin (green); (d) overlay of CPMV-A647 (red—injected 24 h previously) and CPMV-A750 (yellow—injected before imaging). Nanoparticles injected 24 h apart label quiescent vasculature equivalently. (e–h) Mapping of tumor neovascularization: (e) visualization of vasculature using CPMV-A647 24 h after injection; (f) visualization of vasculature using CPMV-A750 immediately after injection—white arrowheads indicate neovasculature; (g) visualization of human HT-1080 fibrosarcoma tumor (magenta) with the endothelium labeled with fluorescein lectin (green); (h) overlay of CPMV-A647 (red, injected 24 h previously) and CPMV-A750 (yellow, injected before imaging). Nanoparticles injected 24 h apart differentially label neovasculature (white arrowheads).

32 | Fill the outer jacket of the embryo imaging unit with distilled water heated to 37 °C and place the entire unit onto a microscope stage inside an environmental chamber equilibrated to 37 °C (**Fig. 4c**). Fix the embryo imaging unit onto the stage with tape or other adhesive to minimize movement artifacts.

33 | The imaging protocol can be designed to best address the particular experiment. The embryo imaging unit will keep the field of view fixed, allowing both three-dimensional Z stacks and time-lapse images to be captured. Acquisition software with the appropriate three-dimensional and time-lapse packages is strongly recommended. We acquire and analyze three-dimensional time-lapse images using the Perkin Elmer (formerly Improvision) Volocity software package. To visualize the dynamics of blood flow, a rapid succession of fluorescence images are acquired in a single plane (**Supplementary Videos 1–5**). For detailed structural analyses at specific time points, a high-resolution three-dimensional stack is acquired (**Fig. 5**). Detailed structural changes in vasculature are mapped over time by acquiring three-dimensional stacks at regular time points. Refer to refs. 5 and 49 for examples of imaging cancer cells in relation to the surrounding vasculature using this approach.

● **TIMING**

- Steps 1–4, Propagation of CPMV in plants: 3–4 weeks
- Steps 5–19, Purification of CPMV from infected leaves: 10–12 h
- Steps 20–22, Conjugation of CPMV with fluorescent dyes: at least 8 h (optional overnight incubation)
- Steps 23A and 23B, Characterization of CPMV-dye conjugates: Step 23(A), 0.5–1 h; Step 23(B), 2 h
- Step 24A, Preparation and *ex vivo* culture of mouse embryos: 1 h (plus a total of 8–15 d to obtain mouse embryos)
- Step 24B, Preparation and *ex ovo* culture of avian embryos: 1–3 h (plus a total of 8–15 d to culture avian embryos)
- Steps 25–28, Injection of fluorescently labeled viral nanoparticles: 1 h
- Step 29A, Injection into yolk sac vein of mouse embryo: 30 min
- Step 29B, Intracardiac injection of mouse embryo: 30 min
- Step 29C, Injection of labeled viral nanoparticles for tumor vascular mapping: 0.5–1 h
- Steps 30–33, Intravital imaging: hours to days, depending on the experimental plan

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Steps	Problem	Possible reason	Solution
24B(vii)	Avian embryos become contaminated in culture	Water levels are too low in containers	Remove contaminated embryos. Clean embryos and hatcher thoroughly with bleach. Fill containers with 1 cm of fresh distilled water
24B(xviii)	Difficulty injecting tumor cells	Microinjection needle is blocked	Many tumor cells will rapidly aggregate. Vortex and resuspend cells, replace microinjection needle and increase bore size slightly
24B(xx)	Avian embryos die shortly after tumor implantation	Equipment is contaminated	Ensure all equipment is thoroughly sterilized before tumor implantation
29A(iii), 29A(iv), 29B(v), 29C(i)	Difficulty injecting CPMV nanoparticles	Tip is not sharp enough	Replace microinjection needle and ensure new needle has a sufficiently tapered end and narrow bore
29B(v)	CPMV leaks out of heart during intracardiac injection	Needle tip has punctured through heart	Replace microinjection needle; tip of needle can be scored with permanent marker to allow it to be better positioned during injection

ANTICIPATED RESULTS

The viral nanoparticle imaging approaches described here are well suited for the intravital visualization of vascular beds in live animals. The synthesis and purification process of viral nanoparticles labeled with fluorescent dyes is straightforward using *N*-hydroxysuccinimide (NHS) ester chemistry, and one can expect that, after an overnight incubation, up to 120 dye molecules will be attached per virion. If a higher degree of labeling is required, a hydrazone linkage¹⁵ or copper(I)-catalyzed azide-alkyne cycloaddition (click) strategy¹⁶ can be used. The use of fluorescent viral nanoparticles enables the dramatic visualization of mouse embryonic vascular structure in explanted mouse embryos (**Fig. 6**). Analysis of the impact of germline mutations on vascular development can be easily assessed in embryos between E9.5 and E15.5 when they are isolated with the yolk sac and placenta intact and placed in culture. Injection of 10 μg of CPMV nanoparticles into a small venule of the yolk sac results in the rapid circulation and deposition of nanoparticles throughout the embryonic vasculature, allowing the visualization of yolk sac vasculature (**Fig. 6a**) and blood flow (**Supplementary Videos 1 and 2**). Once the yolk sac is carefully removed, intravital imaging of the embryonic vasculature (**Fig. 6b**) and the visualization of fine vasculature at high resolution

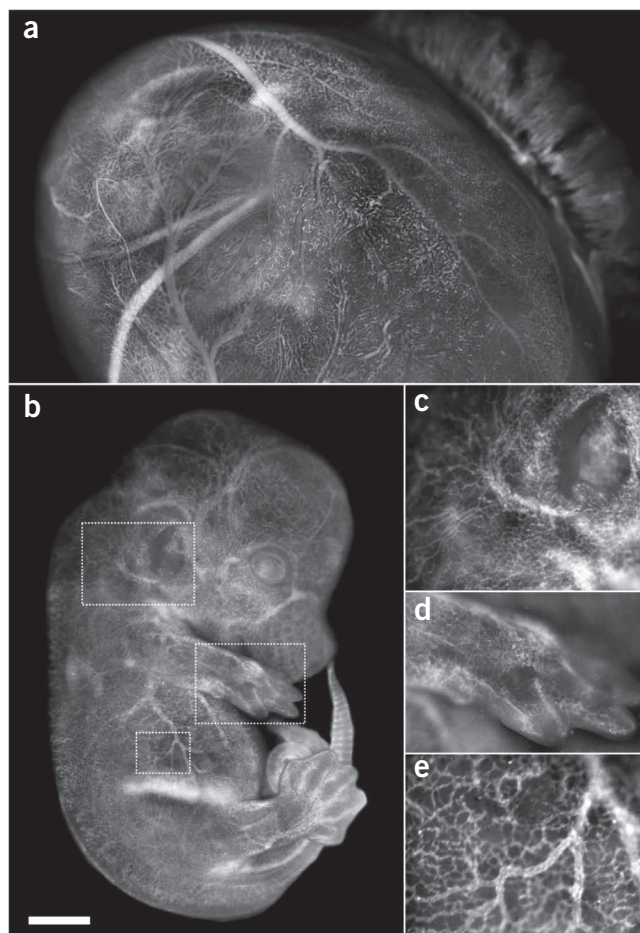


Figure 6 | Intravital imaging of mouse embryo vasculature. **(a)** Fluorescence intravital image of mouse embryo yolk sac vasculature using dye-labeled CPMV. **(b)** Intravital image of mouse embryo vasculature (yolk sac removed). **(c–e)** Magnifications of ear **(c)**, forepaw **(d)** and abdominal **(e)** vasculature of mouse embryo. Scale bar, 1.1 mm.



(Fig. 6c–e) are possible. Microvasculature can be clearly visualized at tissue depths of up to 500 μm, depending on the local tissue density and the extent of nanoparticle labeling. Experiments can incorporate fixation, embedding and sectioning of the tissue of interest with no appreciable loss in fluorescence or changes in biodistribution.

The avian embryo CAM is a well-established model for the study of angiogenesis and tumor growth. CPMV nanoparticles differentially label arterioles and veins in the chick embryo, making their identification at high magnification straightforward, both *in vivo* (Supplementary Video 3) and in tissue sections. When avian embryos bearing highly vascularized human tumors are injected with fluorescent CPMV nanoparticles and visualized by spinning-disk confocal microscopy, the nanoparticles circulate freely through the tumor vasculature, labeling vessels both entering and exiting the tumor as well as the capillary network within the tumor mass. The internalization of CPMV nanoparticles by vascular endothelial cells is especially rapid in the avian embryo, and this facilitates the visualization of vasculature throughout the CAM to a depth of 500 μm. This internalization behavior allows one to differentially label and identify areas of the vascular bed that are involved in active neovascularization (Fig. 5). When viral nanoparticles with different attached fluorophores are injected 24 h apart, the two nanoparticles label quiescent vasculature very similarly (Fig. 5a–d). However, in a tumor-bearing embryo, the two nanoparticles label angiogenic vasculature in a way that allows the identification and visualization of newly formed vessels (Fig. 5e–h). This internalization is significantly inhibited when PEGylated CPMV is used, thus allowing the intravital imaging of blood flow in both preangiogenic (Supplementary Video 4) and vascularized tumors (Supplementary Video 5).

Note: Supplementary information is available via the HTML version of this article.

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AUTHOR CONTRIBUTIONS H.S.L., A.A., J.D.L., A.Z. and H.S. developed the animal models and conducted intravital imaging experiments. N.F.S., G.D. and M.M. prepared and characterized the viral nanoparticles; H.S.L., N.F.S., A.A., H.S., M.M., A.Z. and J.D.L. wrote the paper.

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