Fluorescent Tobacco mosaic virus-Derived Bio-Nanoparticles for Intravital Two-Photon Imaging

Annette Niehl1†, Florence Appaix2, Sonia Boscá1, Boudewijn van der Sanden3, Jean-François Nicoud4, Frédéric Bolze4 and Manfred Heinlein1*

1 Institut de Biologie Moléculaire des Plantes (IBMP-UPR2357), Centre National de la Recherche Scientifique, Strasbourg, France, 2 Two-Photon Microscopy Platform, Grenoble Institut des Neurosciences, Institut National de la Santé et de la Recherche Médicale U836, Université Grenoble Alpes, Grenoble, France, 3 CInC nanoGUNE, Spain, 4 Laboratoire de Conception et Application de Molécules Bioactives, UMR 7199 Centre National de la Recherche Scientifique-Université de Strasbourg, Illkirch, France

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*Correspondence:
Manfred Heinlein
manfred.heinlein@ibmp-cnrs.unistra.fr

† Present Address:
Annette Niehl, Botany, Department of Environmental Sciences, University of Basel, Basel, Switzerland

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INTRODUCTION

Viruses are intensely studied since Tobacco mosaic virus (TMV) was found to be the causing agent of mosaic disease in tobacco (Harrison and Wilson, 1999; Scholthof, 2008). For the vast majority of the about 130 years after this finding, plant viruses have been investigated with the aim to understand and control virus-induced diseases in agricultural crops. As a result of these studies we have learned about the mechanisms of the disease processes and the fundamental nature of viruses, including the composition and structure of the viral particles, the viral replication cycle, and important virus:host interactions (e.g., Zaitlin and Palukaitis, 2000; Ding and Voinnet, 2007; Heinlein, 2015). Since about 30 years, plant viruses are also objects of biotechnological approaches, which led to a broad range of applications, from crop improvement to protein production systems. Plant virus systems have been particularly attractive for the production of recombinant proteins, including biopharmaceuticals, vaccines, and industrial proteins (Scholthof et al., 1996; Pogue et al., 2002; Cañizares et al., 2005; Grill et al., 2005; Gleba et al., 2007). The rod-shaped TMV and the icosahedral Cowpea mosaic virus (CPMV) are among the major vectors for plant-supported mass production of therapeutic peptides and proteins (Haynes et al., 1986; Gallie et al., 1987; Turpen, 1999; Smith et al., 2009; Sainsbury et al., 2010).
More recently, plant and other viruses became recognized as useful templates and building blocks for the development of nanotechnological applications (Steinmetz, 2010). The size of viral particles indeed falls into the nanometer range and thus they can be used for a variety of applications to create novel nano-sized materials with distinct properties. For example, the rod-shaped particles of TMV can be used as templates for mineralization and metallization reactions and the fabrication of highly ordered hybrid materials, even functional devices and ferrofluids (Dujardin et al., 2003; Flynn et al., 2003; Suçi et al., 2006; Tseng et al., 2006; Wu et al., 2010; Atanasova et al., 2011; Chen et al., 2011; Lee et al., 2012). Moreover, virus-derived nanoparticles represent naturally occurring nanomaterials that are both biocompatible and biodegradable, they are also being developed for biomedical applications. Here, plant viruses are particularly attractive since, unlike animal or human viruses, they do not cause diseases in humans. To establish the desired properties for biomedical use, viral nanoparticles (VNPs) can be designed and engineered using both genetic and chemical protocols. By both chemical and genetic manipulation, the viral coat can be tailored to a desired cell or tissue type, imaging modality, or therapeutic cargo. Its multivalent nature enables the incorporation of multiple functionalities, for example targeting ligands and imaging agents, on a single platform (Young et al., 2008), which may lead to potential applications in targeted imaging and therapy (Steinmetz, 2010).

The molecular structure and the biophysical properties of the rod-shaped TMV particle are well known (Alonso et al., 2013). The particle is about 300 nm in length and 18 nm in diameter, with a 4-nm wide inner channel. It has a mass of 39600 kDa and consists of 2130 viral coat protein (CP) subunits encapsidating a 6.7 kb long RNA in a helical arrangement. The CP consists of 158 amino acids and has a calculated mass of 39600 kDa and consists of 2130 viral coat protein (CP) subunits encapsidating a 6.7 kb long RNA in a helical arrangement. Thus, CP monomers carrying a short stretch of 432 nucleotides of its RNA (OAS, origin-of-assembly) being sufficient for assembly (Steinmetz, 2010; Tseng et al., 2006; Wu et al., 2010; Atanasova et al., 2011; Chen et al., 2011; Lee et al., 2012). Moreover, TPLSM employs the excitation of fluorophores by photons in the infrared region for which biological material is transparent (Helmchen and Denk, 2005). Moreover, TPLSM produces background-free images with reduced photobleaching and photodamage since the simultaneous absorption of the two photons required for excitation is intrinsically restricted to the focal point of the excitation beam due to the non-linear nature of TPA (Rubart, 2004). Here, we report the production of TMV particles carrying a two-photon fluorophore and their application as VNPs in intravital imaging of the mouse brain vasculature. The fluorescent signal emitted from the VNPs is stable and does not show any leakage into the surrounding tissues. The particles may have potential to contribute to the noninvasive detection and visualization of pathological alterations in the brain vasculature.

**MATERIALS AND METHODS**

**BF3 Synthesis**

BF3-NCS has been prepared as described (Hayek et al., 2007a). BF3-NCS differs from compounds described in Hayek et al. (2007a) only by a shorter length of the oligoethylene glycol chains. The physico-chemical properties of BF3-NCS are: 1H-NMR, CDC13; (δ ppm): 7.67–7.68 (dd, 2H, J1 = 0.88 Hz, J2 = 8.33 Hz), 7.50–7.46 (m, 4H), 7.11 (s, 2H), 7.06 (s, 2H), 6.80 (s, 2H), 6.78 (s, 2H), 4.24 (t, 4H, J = 4.8 Hz), 4.18 (t, 2H, J = 4.8 Hz), 4.03 (t, 2H, J = 6.3 Hz), 3.92 (s, 6H), 3.89 (s, 6H), 3.87–3.64 (m, 22H), 3.57–3.54 (m, 6H), 3.38 (s, 3H), 3.37 (s, 6H), 2.04–1.85 (m, 8H), 0.70 (s, 10H). 13C-NMR, CDC13; (δ ppm): 152.75; 152.37; 151.49; 140.58; 140.47; 138.32; 137.50; 136.68; 136.18; 136.10; 133.43; 133.06; 128.79; 128.68; 127.86; 127.74; 125.59; 120.62; 119.85; 106.19; 105.37; 103.44; 77.18; 72.37; 72.07; 71.92; 71.90; 71.89; 70.80; 70.74; 70.68; 70.66; 70.54; 70.51; 70.50; 70.45; 69.75; 68.83; 68.73; 58.99; 56.06; 55.93; 44.79; 43.79; 26.99; 26.85; 17.15; 14.48. C63H87NO15S HRMS: m/z 1129.57952 (calc. m/z 1129.57963).

**Isolation of TMV Particles**

TMV particles were purified as described previously (Niehl et al., 2012). Briefly, leaves of TMV-infected *Nicotiana benthamiana* plants were ground in liquid N2 to fine powder. After addition of 10 mM sodium-phosphate buffer pH 7.2 containing 0.1% 2-mercaptoethanol, the viral particles were extracted with 1 volume of butanol/chloroform (1:1), precipitated with 4% polyethylene glycol (PEG) 8000, resuspended in 10 mM sodium-phosphate buffer pH 7.2 and cleared by centrifugation at 5000 × g for 10 min. After two cycles of precipitation in 4% PEG and clearance, the particles were resuspended in 10 mM sodium-phosphate buffer pH 7.2 and stored at −20°C until further use.

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**NMR, CDCl3;**

- δ ppm: 7.67–7.68 (dd, 2H, J1 = 0.88 Hz, J2 = 8.33 Hz), 7.50–7.46 (m, 4H), 7.11 (s, 2H), 7.06 (s, 2H), 6.80 (s, 2H), 6.78 (s, 2H), 4.24 (t, 4H, J = 4.8 Hz), 4.18 (t, 2H, J = 4.8 Hz), 4.03 (t, 2H, J = 6.3 Hz), 3.92 (s, 6H), 3.89 (s, 6H), 3.87–3.64 (m, 22H), 3.57–3.54 (m, 6H), 3.38 (s, 3H), 3.37 (s, 6H), 2.04–1.85 (m, 8H), 0.70 (s, 10H).
- 13C-NMR, CDC13; δ ppm: 152.75; 152.37; 151.49; 140.58; 140.47; 138.32; 137.50; 136.68; 136.18; 136.10; 133.43; 133.06; 128.79; 128.68; 127.86; 127.74; 125.59; 120.62; 119.85; 106.19; 105.37; 103.44; 77.18; 72.37; 72.07; 71.92; 71.90; 71.89; 70.80; 70.74; 70.68; 70.66; 70.54; 70.51; 70.50; 70.45; 69.75; 68.83; 68.73; 58.99; 56.06; 55.93; 44.79; 43.79; 26.99; 26.85; 17.15; 14.48.

**C63H87NO15S HRMS: m/z 1129.57952 (calc. m/z 1129.57963).**
Dye Coupling and Purification of TMV-BF3

300 µg of the purified viral particles per mg BF3-NCS dye were suspended in a sodium-phosphate buffer pH 7.2—DMSO mixture (3:1 V/V) and incubated in the dark at room temperature for 1.5 days. After coupling, the TMV-BF3 particles were separated from unbound dye and concentrated by centrifugation through Microcon YM-10 size exclusion columns (Millipore) using the manufacturer's instructions. The quality and quantity of the concentrated TMV-BF3 particles was assessed by transmission electron microscopy (TEM) following staining with 2% uranyl acetate and by SDS-polyacrylamide electrophoresis (SDS-PAGE) using 15% SDS-polyacrylamide gels. Gels were imaged under UV light using an EttaN DIGE imager (GE Healthcare) equipped with a Sypro2 (excitation 390/20 nm; emission 595/25 nm) filter. After imaging, gels were stained with R250 Coomassie brilliant blue.

Photometric Measurements

All UV-visible absorption spectra were recorded on a Hitachi U3000 spectrophotometer in a dual beam mode using a matched pair of 1 x 1 cm quartz cells. Pure solvent was used as reference. Fluorescence emission spectra were measured with optically diluted solutions (Abs. < 0.15) in 1 x 1 cm cells using a Photon Technology International, Inc. (PTI) spectrofluorimeter.

Two-Photon Absorption (TPA) Measurements

The TPA cross-section spectra were obtained by up-converting fluorescence using a neodymium-doped yttrium aluminum garnet Nd:YAG-pumped optical parametric oscillator that produces 2.6 ns [full width at half maximum (FWHM)] pulses for excitation in the 450–650 nm spectral range and a Ti:sapphire femtosecond laser for excitation in the 700–900 nm range. This set-up does not allow TPA measurements between 650 and 700 nm. The excitation beam was collimated over the spectrophotometric cell length (10 mm). The fluorescence, was collected at 90° of the excitation beam and focused into an optical fiber connected to a spectrometer. The incident beam intensity was adjusted to ensure an intensity-squared dependence of the fluorescence over the whole spectral range. Calibration of the spectra was performed with p-bis(o-methylstyril)benzene, for which the TPA cross section σ2 is 70 GM (Göppert-Mayer) at 570 nm (1 GM = 10−50 cm4 s / photon), and with Rhodamine B, for which the TPA spectrum at 700–900 nm is known (Xu and Webb, 1996).

Two-Photon Fluorescence Correlation Spectroscopy (TP-FCS)

TP-FCS was performed with a home-built setup. TP excitation was provided by a Tsunami Ti:sapphire laser pumped with a Millennia V solid-state laser (Spectra-Physics, Mountain View, CA). 80 MHz pulses of 100 fs were applied with a wavelength of 760 nm. Following passage through a beam expander, the infrared light was focused into the sample by a water-immersion Olympus objective (60×, NA = 1.2) mounted on an Olympus IX70 inverted microscope. The back aperture of the objective was slightly overfilled, creating a diffraction-limited focal spot. The sample and the reference dye were placed in eight wells of a Lab-Tek chambered cover glass (Nalge Nunc International, Rochester, NY) positioned in the X and Y directions by a motorized stage (Märzhäuser, Germany). The fluorescence from the samples was collected through the same objective and directed by a COWL750 dichroic mirror (Coherent, Orsay, France) toward a 50 µm diameter optical fiber coupled to an avalanche photodiode (SPCM 200 FC, EG&G, Canada). Residual infrared light was rejected by a BG39 Filter (Coherent). For FCS measurements, the normalized autocorrelation function (ACF), G(τ), of the fluorescence intensity fluctuations was calculated online by an ALV5000E digital correlator card (ALV, Langen, Germany). Calibration of the system was performed with a 50 nM tetramethylrhodamine (TMR) solution. Assuming a diffusion constant of 2.8 × 10−10 m2 s−1 (Clamme et al., 2003), the equatorial (r0) and axial (z0) radii of the focal volume were, respectively, 0.29 and 1.3 µm, giving an effective volume of 0.2 fL. Assuming a three-dimensional Gaussian distributed excitation intensity, the ACF curve for our sample was fitted as described by Clamme et al. (2003). The fitting curve for our sample corresponded to a bi-exponential function (y = Aeαx+B + Beβx+C), thus reflecting the presence of two different species with very distinct molecular weights (BF3-coupled virus particles and free BF3 dye) in the solution.

In vivo Two-Photon Laser Scanning Microscopy (TPLSM)

In accordance with the policy of Grenoble Institute of Neuroscience (GIN) and the French legislation, experiments were done in compliance with the European Community Council Directive of November 24, 1986 (86/609/EEC). The research involving animals was authorized by the Direction Départementale des Services Vétérinaires de l’Isère—Ministère de l’Agriculture et de la Pêche, France and the Direction Départementale de la protection des populations—Préfecture de l’Isère-France (F. Appaix, PhD, permit number 38 09 39). All efforts were made to minimize the number of mice used and their suffering during the experimental procedure. CD1 Mice were housed in cages with food and water ad libitum in a 12 h light/dark cycle at 22 ± 1°C.

For in vivo TPLSM, the 4 months old CD1 mouse in the experiment was anesthetized using isoflurane (5% for induction and 1–2% during experiments) in a 70% air, 30% O2 gas mixture. Its body temperature was monitored with a rectal probe and maintained at 36°C using a heating blanket. The MouseOx system (STARR Life Sciences Corp.) was used for monitoring arterial O2 saturation, as well as the heart beat and breathing rate. A catheter (NeoflonTM, BD, USA) was inserted in the tail vein for intravenous (iv) injection of 100 µL of TMV-BF3 (50 mg/mL) in saline just before the imaging experiments. Sulphorhodamine B was diluted to 10 mg/mL in saline and 0.1 mL was injected 1 h after the iv injection of TMV-BF3 in order to control if the fluorescent TMV particles addressed the same brain microvessels as observed by small conventional dyes like Sulforhodamine B.

For intravital two-photon imaging of the cerebral vasculature, a craniotomy of 2–3 mm in diameter was performed with a
surgical drill above the motor cortex and filled with ultrasound gel (the head was fixed in a homebuilt stereotactic frame). TPLSM was performed using a LSM 7 MP (Zeiss, Germany) equipped with a 20x water-immersion objective (NA 1.0; Zeiss) and ZEN 2010 software. The blue fluorescence light emission of the TMV-BF3 particles and the red fluorescence of the sulfonrhodamine B dye were simultaneously collected in the epifluorescence configuration using two photomultiplier tubes with a FF01 492/SP25 nm filter (Semrock, US) in front of the "blue PMT" and a FF01 617/73 nm filter (Semrock, US) in front of the "red PMT." Laser excitation was performed at 800 nm using a Ti:Sapphire laser (Chameleon Vision II; Coherent, UK). All the TPLSM images were obtained with less than 50 mW laser power at the cortical surface. Most 3D TPLSM images were acquired as z-stacks with 607 × 607 μm size x-y direction and 2 μm step sizes between each z-focus plane. The 3D projections were performed with Fiji software (http://fiji.sc/Fiji) using the in-built "Z-Project" feature (http://fiji.sc/Z-functions) and the standard deviation projection method.

RESULTS

Preparation of BF3-Coupled TMV Particles

TMV particles isolated from infected Nicotiana benthamiana plants were coupled to BF3-NCS (Hayek et al., 2007a), a derivative of the two-photon-excitable fluorophore BF3 (Hayek et al., 2006; Figure 1A), a non-toxic molecule emitting blue fluorescence with an improved fluorescence quantum yield of 73% in water. BF3-NCS carries long oligoethylene glycol side chains to provide increased water solubility and an iso-thiocyanate group (NCS) for chemical coupling (e.g., to −NH2 and −OH groups, i.e., the NH2 group at the N-terminus or the −OH group of tyr 139 of CP). As a one-dimensional (1D) conjugated molecule of the general symmetrical D–conjugated π system−D (D = electron donor group) structure, it shows a high TPA cross-section (Figure 1D).

Before conjugation, virus particles were purified from TMV-infected plants. The purity of the viral particle preparation was assessed by SDS-PAGE. The particles were then conjugated to BF3-NCS in a water/DMSO mixture for 36 h and purified by size exclusion centrifugation. As compared to the colorless solution that contains viral particles before coupling or the pale-yellow color of the solution containing coupled but non-purified particles, the solution containing the concentrated BF3-coupled viral particles was characterized by a strong yellow color (Figure 1B) and showed intense blue fluorescence in UV light. Following coupling and purification, the viral particle preparation maintained infectivity as was verified by the observation of local cell death lesions forming on leaves of hypersensitive Nicotiana tabacum NN tester plants (not shown). The typical rod-like shape and size dimension of the TMV particles was not affected by BF3-coupling as was confirmed by electron microscopy (EM; Figure 1C). 1-photon absorption and fluorescence emission spectra of the BF3-conjugated particles showed maxima at 382 and 452 nm, respectively. Moreover, the absorption was in agreement with the two-photon absorption peak of the free dye (Figure 1D). These values are similar to the spectra previously reported for the parent BF3 molecule (Hayek et al., 2007b). Analysis of the purified particles by SDS-PAGE revealed a fluorescent protein band running at higher molecular weight (approximately 18.5 kD) than the normal CP (17.6 kD), thus confirming successful conjugation (Figures 1E,F).

The two-photon sensitivity as well as the purity of fluorescent particles were verified by two-photon fluorescence correlation spectroscopy (TP-FCS), which showed a long diffusion time of 0.75 ms corresponding to the labeled viral particle (>95%) and a shorter time of 0.024 ms, which corresponds to free dye (<5%) (Figure 1G).

Application of TMV-BF3 Particles for Intravital Imaging

To determine the applicability of the dye-conjugated particles for in vivo TPLSM imaging, we injected 100 μL of the particle solution [50 mg/mL in saline (0.9% NaCl)] into the tail vein of an anesthetized 4 months-old CD1 mouse placed on the microscope stage and prepared by craniotomy. We then observed the cerebral vasculature at the surface of the motor cortex by TPLSM using a high numerical aperture, 20x water immersion objective and an excitation wavelength at 800 nm. The cerebral vasculature was readily visible by strong fluorescence with no obvious evidence for leakage until 1.5 h after iv injection (see Figures 2A,B). In previous studies (Hayek et al., 2007b), the free fluorescent dye diffused freely across the BBB in an 8 months old mouse within 20 min after injection. Acquisitions of z-stacks were used to reconstruct 3D animations that provide detailed views of the brain vasculature (Movie 1). To verify the pattern of blood vessels seen with TMV-BF3 by a different staining, the same mouse was post-injected (1 h after injection of TMV-BF3) into the tail vein with 100 μL of a sulfonorhodamine B solution (10 mg/mL). As shown in the overlay Figure 2B, at 1.5 h post-injection the pattern of vessels labeled with the red fluorescent dye (red) overlapped with the pattern of vessels labeled with TMV-BF3 (blue). However, there were also important exceptions of vessels that were labeled with TMV-BF3 but not or, only weakly, with sulfnorhodamine B (e.g., the vessel highlighted by arrows). This observation indicates that the TMV-BF3 particles partly blocked the blood perfusion in small brain vessels.

DISCUSSION

Our observations demonstrate that TMV particles labeled with a multi-photon absorbing fluorochrome can be used as a blood pool dye for deep tissue vascular imaging. Current agents for vascular imaging are based on liposomes, quantum dots, dextrans, lectins, antibodies, iron oxide particles, and nanoparticles. Although each particle type has certain advantages, diverse problems in relation to stability, bioavailability, toxicity, chemical flexibility, and cost exist (Lewinski et al., 2008; Cheng et al., 2012; Adjei et al., 2014). Moreover, inorganic synthetic particles tend to aggregate and may cause toxic effects under physiological conditions (Kirchner et al., 2005). TMV particles represent natural materials that are both biocompatible and
FIGURE 1 | Production and analysis of TMV-BF3 particles. (A) Molecular formulae of BF3 and BF3-NCS. (B) Solutions containing purified TMV-particles before coupling (left), non-purified TMV particles after coupling (middle), and TMV particles size-purified after coupling (right). (C) EM image showing integrity of TMV particles after coupling. (D) Absorption (red) and emission (black) spectra of TMV-BF3 particles in phosphate buffer; two-photon excitation spectrum (blue) of BF3-NCS in water; a.u., arbitrary units; GM, Goeppert-Mayer units. (E,F) Electrophoretic analysis of the size-purified TMV-BF3 particles under denaturing conditions. (E) Coomassie blue-stained SDS-PAGE gel showing the presence of CP (17.6 kD, asterisk). (F) Same gel as in E but analyzed with a fluorescence scanner equipped with a sypro2 filter. A fluorescent band at approximately 18.6 kD is detected and confirms the presence of BF3-coupled CP. The orange dye conjugated to the 70 kDa protein of the size ladder (Thermo Scientific) also exhibits fluorescence under the illumination conditions used. The occurrence of fluorescence below the BF3-CP band suggests that the bond between BF3 and CP is partially sensitive to the denaturing SDS-PAGE conditions. (G) TP-FCS analysis of TMV-BF3 particles under native conditions showing a long diffusion time of 0.75 ms corresponding to the labeled viral particle (>95%) and a shorter time of 0.024 ms corresponding to free dye (<5%).

biodegradable and thus provide a safe platform for biomedical applications. Because of their anisotropic shape and their size, the particles may provide a sensitive probe to measure blood flow. In addition, large dyes also play a role as probes in other cases of brain diagnostics such as the optical mapping of damages to the BBB. The BBB consists of a tightly packed layer of endothelial cells that acts as a physical barrier between the blood vessels and the central nervous system and prevents many substances from diffusing across it (Rubin and Staddon, 1999). Disruption of the BBB causing leakage of substances from the blood vessels into the brain is a serious condition that occurs in many pathological conditions such as brain tumors (Jain, 1987; Dvorak et al., 1988; Hashizume et al., 2000), brain injuries (McDannold et al., 2008; Moretti et al., 2015), infections of the central nervous system (Lossinsky and Shivers, 2004), neurological diseases such as multiple sclerosis (Patel
FIGURE 2 | Intravital imaging of the mouse brain vasculature with TMV-BF3 particles. (A) Mouse brain vessels labeled with TMV-BF3 at 1 h after intravenous injection into the tail vein. (B) Same observation window as shown in (A) but after a second injection, this time with sulforhodamine B; blue, fluorescence emitted from TMV-BF3; red, fluorescence emitted from sulforhodamine B. The 3D projections were performed with Fiji software using the standard deviation projection method.

The utilization of plant VNPs for intravital vascular imaging was previously demonstrated with particles of *Cowpea mosaic virus* (CPMV; Lewis et al., 2006). However, unlike in our current study, one-photon dyes were used and fixation and sectioning were required for deep tissue imaging in adult mice. Moreover, the icosahedral particles of CPMV do not provide flexibility in particle design like TMV. Interestingly, the fluorochrome-tagged CPMV particles did not remain within vessels but entered the vessel endothelium. Although such feature may contribute to the long-term stability of the fluorescence signal, it also limits application in blood flow measurements and may also enhance the risk of leakage across the BBB. In addition to CPMV and TMV, many other plant viruses are being investigated as VNPs for applications in medicine (Steinmetz et al., 2010; Yildiz et al., 2010; Wen et al., 2012; Shukla et al., 2014). For example, CPMV and other plant viruses, like *Cowpea chlorotic mottle virus* (CCMV), and also bacteriophages such as MS2 and Qβ, are explored as platforms for the binding of several hundred contrast agents in order to improve the efficiency of magnetic resonance imaging (MRI; Liepold et al., 2007). Combining these or fluorescent plant VNPs (e.g., TMV-BF3) with specific targeting ligands and therapeutic agents such as drugs or peptides may lead to the development of novel cost-effective tools for *in vivo* imaging and treatment approaches that may eventually revolutionize the current concepts of diagnosis and therapy.
AUTHOR CONTRIBUTIONS
AN, FB, JFN, BS, and MH conceived and designed the work; AN, SB, FA, and FB performed the acquisition and analysis of the data; AN, FB, JFN, BS, and MH interpreted the data; AN, FA, SB, BS, JFN, FB, and MH drafted the work; FB, BS, and MH wrote the manuscript and revised it critically for important intellectual content; AN, FA, SB, BS, JFN, FB, and MH approved the final version to be published and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.01244

Movie 1 | 3D-animated part of the TMV-BF3 labeled mouse brain vasculature.

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