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Low Magnification Confocal Microscopy of Tumor Angiogenesis

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Abstract

Blood vessels are critical to normal mammalian development, tissue repair, and growth and treatment of cancer. Mouse research models enable mechanistic studies of blood vessels. We detail how to perfuse mice with fluorescent tomato lectin or the lipophilic fluorophore DiI. We provide details on how to image fluorescently labeled blood vessels.

Key words Blood vessels, Angiogenesis, Fluorescent lectin, DiI, Confocal microscope

1 Introduction

Inhibiting tumor growth with anti-angiogenic therapies has received much interest because of the work by Judah Folkman [1]. Blood vessels, and blood flow, can now be visualized in vivo at depths to ~1 mm in mouse tissues using intravital microscopy with expensive multiphoton excitation laser microscopes (see refs. 2–6). Not having a multiphoton excitation laser, and because our tumors were implanted several millimeters deep in mouse brains, we have adopted an alternative approach of quantifying tumor growth by in vivo bioluminescence and MicroMRI imaging, and then performing visible and near-infrared confocal microscopic imaging of tumors in hemi-sectioned mouse brains. We were inspired to use a 10× objective lens for this project, by the work of R.M. Zucker with low-magnification confocal microscopy of mouse and rat fetuses [7–9]. We used fluorescent tomato lectin for our work, but note that Debbage and colleagues have obtained excellent staining of blood vessels, both by perfusion and in tissue sections, using any of several other lectins [10–12].
2 Materials

Female athymic nude mice (nu/nu, albino outbred stock from NCI), 6–8 weeks of age (Harlan, Indianapolis, IN).

U87MG human glioblastoma cell line (ATCC, Manassas, VA).

hrLuc-DsRed2-sr39tk HSV TK tribrid gene (Ray et al. [20]; S.S. Gambhir, pers. comm.).

RPMI with 10 % FBS in 5 % CO₂ (GIBCO™ Invitrogen Corp., Carlsbad, CA).

Fluorescein-conjugated tomato lectin (Vector Laboratories, Burlingame, CA).

Biotin-conjugated tomato lectin (Vector Laboratories, Burlingame, CA).

Alexa Fluor® 647-streptavidin (Molecular Probes, Inc., Eugene, OR).

Glass bottom culture dishes, P35G-0-14-C or P35GC-1.5-14-C (Mattek Corp., Ashland, MA).

Leica TCS SP1 confocal microscope optics mounted on a Leica DMIIRBE inverted microscope; LCS and LCS Lite confocal microscope acquisition and analysis software (Leica Microsystems, Exton, PA and Heidelberg, Germany). 2009 (U Miami): Leica SP5 spectral confocal DMI6000 inverted microscope or Zeiss LSM510/UV confocal Axiovert 200M inverted microscope.

Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA).

Anti-PECAM-1 (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA).

Anti-Smooth Muscle Actin Clone 1A4 (Dakocytomation, Carpenteria, CA).

Cy3-conjugated AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen, San Diego, CA).

Biotinylated anti-rat IgG (VECTOR Laboratories, Burlingame, CA).

Hoechst 33342, H3570 (Invitrogen/Molecular Probes), 10 mg/mL stock solution, diluted to 100 μg/mL in distilled H₂O, used at 0.1 μg/mL (live cell experiments) or 10 mg/mL in PBS (hemi-sectioned brain nuclear labeling).

Leica Microsystems (Bannockburn, IL) DMRXA fluorescence microscope (see text for additional details).
Pathscan Enabler III (in 2008 version IV) (Meyer Instrument, Houston, TX).
Polaroid SprintScan 4000+ (Polaroid Corp., Cambridge, MA).
Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA).

3 Methods

The methods described below outline animal injection, sacrifice, brain confocal imaging, and immunofluorescent analysis of tissue sections. The tribrid reporter gene is described in Ray et al. [20]. Details of other methods can be found in other publications [13, 14, 19, 21–26]. Athymic nude (nu/nu) immunocompromised female mice, 6–8 weeks of age (Harlan, Indianapolis, IN), are used as the recipients of human tumor cells.

3.1 Tribrid Reporter Gene

A multipurpose reporter gene was introduced into the U87MG glioblastoma cell line by lentivirus transduction [22]. The hrLuc-RFP-TK tribrid gene [20, 27], courtesy of Sam Gambhir, UCLA (now at Stanford University), is a triple fusion of human codon-optimized Renilla luciferase (hrLuc), DsRed2 red fluorescent protein (RFP) (BD Clontech) and mutant sr39tk herpes simplex virus type 1 thymidine kinase (TK) (Ray et al. [20] for the precursor Rluc-sr39tk hybrid gene fusion article). Note that Ray et al. [20] report on an improved tribrid vector that replaces the DsRed2 RFP with a monomeric DsRed derivative (mRFP1, Campbell et al. [28]) and R.Y. Tsien’s lab has made spectral and brightness improvements to mRFP1 in their fruity fluorescent protein series of papers [29, 30].

Rluc enables whole animal in vivo bioluminescence imaging with a Xenogen 3D IVIS® imaging system for quantitation of tumor cell mass following native coelenterazine injection (collaboration with Mike Rosol and Maya Otto-Duessel, CHLA). In this and related studies, tumor size was also estimated by T1- or T2-weighted or contrast enhanced (tumor tissue edema) MicroMRI using either a custom mouse coil on a clinical MRI machine (GE Medical Systems 1.5 Tesla MRI) or on a 7 Tesla Bruker MicroMRI (collaboration with Rex Moats, Harvey Pollack and Maya Otto-Duessel, CHLA). MRI cross-section area correlates well with histology area [22, 31].

The DsRed2 red fluorescent protein (RFP) enables whole mount confocal microscopy and fluorescence stereomicroscope (Leica MZ FL III) of split brains, fluorescence microscopy of tissue sections by DsRed2 fluorescence or anti-DsRed immunofluorescence or immunohistochemistry with a compound microscope.
(Leica DM RXA), and flow cytometry (BD Biosciences FACSCalibur) of trypsinized brain. The tumor cells are labeled with the tribrid hrLuc-RFP-TK fusion triple reporter because we wanted to reserve the more sensitive firefly luciferase (Fluc) in vivo reporter for therapeutic stem cell tracking [22, 29, 32, 33].

The TK gene product uses a ganciclovir substrate, which the herpes simplex virus mutant sr39tk thymidine kinase converts to a toxic product, and as a PET reporter (MicroPET collaboration with Xiaoyuan (Shawn) Chen, USC PET Imaging Science Center).

Mice were orthotopically injected with $10^5$ U87 glioblastoma cells. Mice were anesthetized using ketamine (Ketajet 100 mg/kg) and xylazine (Xyla-ject 10 mg/kg) and $1 \times 10^5$ U87 MG glioblastoma cells in 1 μL of serum free medium were inoculated in 20 min stereotactically in the defined location of the caudate/putamen (0.5 mm anterior to the bregma, 2.0 mm lateral to the midline) using a 10 μL Hamilton syringe (Reno, NA) advanced to a depth of 3.3 mm from the cortical surface. The slow rate of 100,000 cells in 1 μL in 20 min was necessary for the tumor cells to remain deep in the brain tissue. Rapid infusion, or rapid drawback of the injection needle, can result in the tumor cells becoming dispersed along the needle track and rapid growth along the track and along the surface of the mouse brain (S. Yamada, V. Khankaldyyan, W. Laug, R. Moats, I. Gonzalez-Gomez, unpublished MicroMRI and histology data). A full stereotaxis rig maximizes consistency of the xenograft model (M. Rosol, M. Jensen, pers. comm.).

3.3 Tomato Lectin Intravital Labeling of Blood Vessels (Vascular Casting)

We used either 1 mg/mL fluorescein (isothiocyanate) conjugated tomato (Lycopersicon esculentum) lectin (Vector Laboratories), or Alexa Fluor®-streptavidin (Molecular Probes) mixed immediately before use with biotin-conjugated tomato lectin (Vector Laboratories). Tumor-bearing mice were anesthetized and injected by cardiac puncture using a 28 gauge needle with 200 μL of fluorescent tomato lectin in PBS. Two minutes later, the mice were perfused with PBS intracardially to flush out red blood cells. Best results are obtained after maximally flushing out the red blood cells. An alternative to PBS perfusion (not used in the current work) is freshly prepared 4% paraformaldehyde in PBS. This has the advantage of making the tumor tissue stiffer for semi-sectioning, but has the disadvantage of increasing tissue auto-fluorescence, greatly increasing red blood cell auto-fluorescence, and disruption of some antigenic epitopes used for later immunofluorescence or immunohistochemistry. An alternative to cardiac puncture is tail vein injection. For lectin staining of brain microvessels, we obtained more consistent labeling with cardiac puncture. We have on occasion harvested other organs (liver, spleen, kidney/adrenal gland, lungs) from mice in this study (A. Yanai and V. Khankaldyyan) or from neuroblastoma orthotopic implantation or tail vein injection.
metastasis models (Chantrain et al. [34]; Y. DeClerck, C. Chantrain, K. Bajou, L. Sarte, S. Jodele, pers. comm.) after cardiac or tail vein injections. Cardiac puncture has a clear advantage for brain microvessel labeling, due to the “straight shot” from the aorta to the carotid arteries and brain blood vessels. Tail vein injection works somewhat better for the other organs, for those mice that have good tail veins and available injection sites following repetitive injections of chemotherapies or bioluminescent substrates. The neuroblastoma group has also evaluated Texas Red®-streptavidin tomato lectin for microvessel imaging with similar performance to those reported here (DeClerck et al. and McNamara, unpublished). Animal protocols were performed with CHLA animal care committee and biosafety committee approval. We anticipate future experiments that will obtain similar or superior data with fluorescent nanocrystals (aka quantum dots), i.e., QD655-streptavidin and/or QD705 streptavidin (Quantum Dot Corp., http://www.qdots.com) or Evitag720-streptavidin (Evident Technologies, http://www.evidenttech.com), as has been published for blood (Larson et al. [3]) and lymph node (Kim et al. [35]) imaging in live animals [35].

3.4 Brain Excision and Hemi-sectioning

For confocal imaging, brains were excised and sectioned in half (horizontal section). Horizontal sectioning was chosen because the U87MG-tribrid gene transfected human glioblastoma tumor cells were orthotopically injected in the middle of the mouse brain [21, 22, 26, 31]. The hemi-section was made with a scalpel, the cut being from the olfactory bulb to cerebellum, approximately bisecting the orthotopic implanted tumor. This is the same plane as one of the axes of the MicroMRI 3D scans [31], and is also used for histological sections by fluorescence immunohistochemistry and standard immunohistochemistry with either light microscopy (see below) or 35 mm film/Pathscan microscope slide scanner and image analysis [21, 34]. The brain halves were placed in cold PBS on ice, transported to the confocal microscope, transferred to a uncoated #0 or #1.5 coverglass 35 mm glass bottom dish (P35G-0-14-C, or P35GC-1.5-14-C, Mattek Corp., http://www.glassbottomdishes.com/gbcustomerpriceweb.pdf). Following confocal imaging, the brain hemi-sections were put back on ice, transported back to the wet lab, fixed, sectioned and then individual sections were processed for H&E histology, immunofluorescence and immunohistochemistry. In collaboration with Dr. Christine Brown, Renate Starr and Professor Michael Jensen, we have imaged Hoechst 33342 dye nuclear counterstaining of hemi-sectioned mouse brains, at the Light Microscopy Core of City of Hope National Medical Center. Brain nuclei were imaged on a Zeiss LSM 510 NLO confocal/multiphoton microscope in PBS with Hoechst 33342 at 10 μg/mL for 15 min, transferred to an imaging dish with PBS, and imaged using 750 nm, ~80 MHz,
~100 femtosecond pulses with a Zeiss 10×/0.5 NA lens. On the same microscope we have performed live cell experiments with Hoechst 33342 at 0.1 μg/mL in bicarbonate free, phenol red free, tissue culture medium. This concentration was chosen because higher concentrations of Hoechst are known to inhibit normal cell behavior [37]. When using Hoechst dyes, it is important to dilute the 10 mg/mL stock solution 100-fold in water because diluting in PBS results in formation of dye precipitates.

Details of the CHLA confocal microscope hardware can be found in the Appendix. The Leica SP1 confocal spectrophotometry hardware has been described [38, 39]. This and other spectral confocal microscopes is also available [40], where the CHLA system is system L4.

Most Images were acquired with Leica TCS SP1 confocal optics, equipped with air-cooled Argon ion (457, 476, 488, and 514 nm laser lines, ~2 mW power at 488 nm at the specimen plane), air-cooled Krypton ion laser (568 nm, ~2 mW power) and HeNe laser (633 nm, ~2 mW) mounted on a Leica DM IRBE inverted fluorescence microscope. The Argon and Krypton ion lasers operational lifetime is low if run at full power (maximum power knob setting of 4 o’clock on laser front panel); we routinely operated both at 12 o’clock power settings (mW power shown above for 100 % AOTF settings); when idle, the laser power was set to the minimum (knob setting 8 o’clock). Laser power was attenuated with the Leica SP1 AOTF. The Leica confocal microscope is maintained under an annual service contract with the manufacturer (Leica Microsystems, Exton, PA). In addition to an annual preventive maintenance visit by the manufacturer’s field service engineer, the image core manager performs periodic performance tests and arranges service visits as needed (testing details available from GM). Confocal system performance data, during the time period of image acquisition for this study, was published as system L4 [40].

Three reflection/fluorescence and one transmitted light (through the condenser) photomultiplier tubes (PMTs) are present on our SP1 confocal microscope. For fluorescence, the PMT offset were adjusted such that a positive intensity value was read out even with no light reaching the detector. Typical PMT gains were in the 700–900 settings range (maximum 1,250). A Leica RSP500 or TD488/568/633 triple dichroic mirror was used in the scanhead to reflect laser excitation to the microscope and pass emission photons to the PMTs. The SP1 optical head uses a prism spectral dispersion element with wavelength selection slits in front of each PMT. The SP1 is controlled from Leica LCS 2.5 on a Pentium 400 MHz computer. Confocal images were acquired with no filter cube in place. The Leica DM IRBE microscope stand is equipped Leica A, I3 and N2.1 longpass filter cubes, for DAPI,
fluorescein/GFP/Alexa488, Cy3/DsRed, respectively, excited with a 50 W Hg lamp for visual inspection (a 100 W Hg voltage stabilized lamp would be a better choice for standard microscopy, since the Leica 50 W Hg lamp flickers). Information on filter cubes can be found in Ploem and Walter [41]. We occasionally acquired brightfield or Nomarski differential interference contrast (DIC) images with the laser line(s) from the objective lens and specimen being detected through the microscope condenser and a transmitted light path PMT that was positioned adjacent to the microscope transmitted light path, and switchable with a Leica knob. The transmitted light images were not confocal because that light path does not include a pinhole to reject out of focus light; the transmitted light images were in register with the confocal fluorescence. The laser illumination transmitted light DIC images do exhibit optical sectioning because of the intrinsic characteristics of DIC, but the images are not particularly good as the Leica optics exhibit significant shading, the contrast range of the 8-bit images are small, and the thickness of the hemi-sectioned mouse brains scatter much of the light.

Most brain images were acquired with a Leica 10×/0.40 NA HC Plan Apo Ph1 dry objective lens (Leica SP1 confocal microscope). Some images were acquired with a 10×/0.4 NA HC Plan Apo IMM lens, using water immersion, without significant improvement in image quality. We have tested a Leica N Plan 5×/0.12 NA, N Plan 2.5×/0.07 NA, and Plan Fluotar 1.3/0.04 NA objective lenses, for the unfixed hemi-sectioned brain application, but have been whelmed by the fluorescence brightness and contrast. These lenses may perform better using completely transparent, low-scattering, tissue with bright fluorescent markers, such as Zucker’s 1:1 benzyl benzoate–benzyl alcohol (BABB) clearing solution [8] or methyl salicylate clearing solution of brains perfused with red fluorescent latex vascular casts (S. Yamada, CHLA). BABB or methyl salicylate clearing of brain tissue requires ~1 week time after transfer to absolute ethanol, and leaves the tissue fragile with respect to later handling. By contrast, our unfixed brain hemi-section imaging adds a few hours delay between the time the mouse is sacrificed and when the tissue is ready for histological processing.

The confocal pinhole size was routinely set to either 1.0 or 1.5 Airy units when using the 10×/0.40 NA dry objective lens. At 1.0 Airy units, this lens has a theoretical XY resolution of 488 nm and Z resolution of 2,630 nm, with a working distance of 2,200 μm. (The deepest into a fluorescent specimen we have ever imaged was 836 μm with a methyl salicylate cleared red fluorescent latex mouse brain vascular cast.) Opening the pinhole from 1.0 to 1.5 Airy units results in the collection of more photons from the specimen from a thicker optical section. The image capture settings were
1,000 × 1,000 μm field of view (512 × 512 pixels), medium scan speed, 2 or 3 Kalman frame averaging. A Z step size of 2 μm (slightly oversampling in Z) was used to make the XY and Z pixel dimensions equal as a convenience for later image analysis and display (“orthogonal dimensions”). Our Leica SP1 confocal microscope is equipped with a Leica high speed, high resolution galvanometer based focus motor (“galvo-Z”). The galvo-Z enables high speed XZ scanning (at appropriate zoom) at the same speed as standard XY image scanning. The galvo-Z has a step size of ~0.040 μm, range of 160 μm and a mass limit of 90 g. For the purposes of this study, we used the Leica DMIRBE microscope internal focus motor, which has a nominal step size of ~0.100 μm.

Basic Leica SP1 microscope operations are described in a Web document by John Runions at the Haseloff lab (http://www.plantsci.cam.ac.uk/Haseloff/JohnRunions/Confocal_instructions/Confocal_instructions(old)/Confocal.pdf). The Haseloff SP1 system is on an upright microscope and uses different lasers than the CHLA system.

Mouse skin blood vessels were imaged by sacrificing the mouse pinching the skin and cutting the skin scissors. For this work, an approximately 25 mm piece of skin (with white hair), was imaged in a 25 mm Mattek glass bottom dish (P35G-1.5-20-C, http://www.glassbottomdishes.com) with a thin layer of PBS to improve optical contact to the coverglass. Tile scans were acquired with a Leica SP5/DMI6000 inverted confocal microscope (University of Miami). DiI perfused mouse skin blood vessels were acquired with a 561 nm laser, 10×/0.4 NA objective lens, 512 × 512 pixel images with 8 × 6 tile scan Z-series of 21 planes using 10 μm step size (200 μm thick). Non-confocal RGB transmitted light tile scans were acquired on the Leica SP5 microscope using 458, 561 and 633 nm laser lines for blue, green and red channels, respectively, using three scan track sequential frame mode (3× longer time than a single scan track). Because hemoglobin is the major absorbing molecule, in the red blood cells of the mouse that did not perfuse with DiI, we could have used any of the laser lines between 458 and 561 nm for the blue and green channels by applying a cyan lookup table, plus the 633 nm laser line for the red channel. This would have reduced scan time by one third (e.g., 90 min to 60 min for a 21 plane Z-series, 8 × 6 tile scan). For anyone performing transmitted light laser scanning diaminobenzidine and hematoxylin (DAB&H) tissue section slides, we recommend either the 405 or 458 nm laser line for DAB (blue channel) and 633 nm laser line for hematoxylin (green and red channels). Transmitted light laser scanning of hematoxylin and eosin (H&E) slides can performed with transmitted light 633 nm for hematoxylin, and either 488, 496, or 514 nm for eosin, with confocal fluorescence (520–560 nm) and/or non-confocal transmitted light detection (green and blue channel).
3.6 Immunofluorescence and Histological Processing of Tissue Sections

After brains were removed, bisected through the tumor, and imaged on the confocal microscope, they were processed, half for paraffin embedding (Zinc Fixative followed by 10 % formalin fixation) and half for frozen sections (4 % paraformaldehyde followed by 16 h in 30 % sucrose). Tissue for frozen sections were snap frozen in OCT embedding medium and maintained at −80 °C until sectioned. Hematoxylin and eosin stained sections were examined to note extent of tumor growth.

Immunofluorescence for CD31 and αSMA was performed on Zinc fixed paraffin-embedded sections. Five micrometer sections were mounted on Superfrost/Plus glass slides. After baking at 60 °C overnight, the slides were deparaffinized in xylene and rehydrated. For antigen retrieval, sections were boiled 12 min in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). The sections were stained using anti-PECAM-1 (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA) 1:100 and/or anti-Smooth Muscle Actin Clone 1A4 (DakoCytomation) 1:100 and incubated overnight at 4 °C. The secondary antibodies: Cy3-conjugated AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (1:200) and Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (1:200). Frozen sections were stained with purified rat antimouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen) 1:75 Biotinylated anti-rat IgG (VECTOR Laboratories, Burlingame, CA).

3.7 Immunofluorescence Microscopy of Tissue Sections

Tissue immunofluorescence images were acquired with a Leica Microsystems (Bannockburn, IL) DMRXA fluorescence microscope, Sutter Instrument Company (Novato, CA) LS 300 Xenon arc lamp with 1 m liquid light guide, Life Imaging Services (Reinach, Switzerland) LLG-DMRXA quartz lens coupler, Chroma Technology Corp. (Rockingham, VT) HQ filter sets for fluorescein (set 41001), Cy3 (41007a), and Cy5.5 (41022). The microscope was also equipped with a 61000v2 DAPI/Fluorescein/Cy3 triple pass filter set for viewing specimens by eye, and was equipped with additional filter sets for other applications. The DMRXA was equipped with a Leica optovar with 1× (lensless), 1.25× and 1.6× magnifications. Images were acquired using 100 % light to an Applied Spectral Imaging Inc. (Carlsbad, CA) SKY™ SD-300/ VDS-1300 spectral imager, 12-bit digital CCD camera, using EasyFISH software. The camera was usually binned 2×2 to improve the signal to noise ratio, and decrease image exposure time, of the fluorescence images. All images were saved in 16-bit/channel quantitative EasyFISH file format, and in standard 24-bit TIFF format. For microvessel density measurements, slides were scanned at low power (HC Plan 10×/0.4 lens) to identify areas of highest vascularity. Ten to 20 high-powered (HC Plan 20×/0.7 lens)
fields were then selected randomly within these areas, and microvessel densities were calculated based on the number of CD31/biotinylated tomato lectin positive structures. Microvessel counting was performed by multiple blinded observers in conjunction with a pathologist. A pericyte-positive vessel was defined as a CD31-positive vessel surrounded by at least one cell staining positive for α SMA.

For whole-section tumor area quantitation, H&E or immunohistology slides were acquired with a Pathscan adapter (Meyer Instruments) on a Polaroid SprintScan 4000Plus 35 mm slide scanner [23, 42]. See ref. 21 for examples of Pathscanned U87MG orthotopic tumor implantation model mouse brain tissue sections.

Leica Microsystems generously granted us permission to include the LCSLite software on the book CD (also available for free download from ftp://ftp.ltt.de/softlib/LCSLite/ and the newer Leica LAS AF Lite is available for free download at ftp://ftp.ltt.de/softlib/LAS_AF_Lite/). LCSLite operates in Microsoft Windows. The oldest version of LCSLite, v2.0.0871, runs under Windows NT, 2000 and XP. The newer 2.50.1347a and 2.61.1537 only run on Windows NT and XP. Windows Administrator privileges are required for installation. For best results, install on a computer with a dual monitor display and identical monitors. After running LCSLite (or LCS if you are a Leica confocal customer), by default the two channels in the demonstration datasets will open as green and red. Right clicking on the image window will bring up a menu from which you can turn on the LUT control. Clicking on the LUT slider allows you to change to gray or other lookup table color schemes. We recommend grayscale. Positioning the cursor over a LUT slider (but not clicking) results in thumbs being displayed along the top and bottom of the LUT—these allow you to adjust the contrast of a LUT channel. We suggest pulling the upper thumb down to increase the brightness of the DsRed2 channel. The overlay button can be used to display the combined colors.

Leica confocal microscope images are saved in tagged image file format (TIFF) with Z-plane and channel information as part of the filename. For our demonstration datasets, channel 0 (ch00) is PMT1 (fluorescein) and channel 1 (ch01) is DsRed2. Individual TIFF files can be opened in Adobe Photoshop CS, NIH Image, ImageJ, MetaMorph and other image analysis software packages. For ImageJ, we recommend the MBF ImageJ bundle (http://www.macbiophotonics.ca/downloads.htm). For MetaMorph 6.25, we typically copy each channel set to its own subfolder, then use the Image Browser to open each channel as a separate stack. Some images were processed with Adobe Photoshop CS or MetaMorph 6.25 for publications.
The DiI labeling method [13] can also be applied to visualize blood vessels in the lungs or other tissues with the following procedure. An animal is killed by CO₂ overdose or sodium phenobarbital overdose (120 mg/kg, i.p) and then placed on a perfusion stage, positioned on its back. The trachea is exposed and cannulated with a blunt needle of #21-G. Cardiac perfusion is carried out as described (Li et al. [13]), but starts from the right ventricle instead of the left one and drains by cutting the descending aorta instead of the right atrium. The DiI solution is 3 mL with 30 μl of DiI stock solution. After perfusion, lungs are fixed with 4 % paraformaldehyde through the #21-G needle by connecting it to a small reservoir through a line under 20 mm H₂O intrapulmonary pressure (by raising the reservoir to 20 mm above the animal level) for 5–10 min. The lungs then are removed and submerged in 4 % paraformaldehyde for 1 h. Tissue can be viewed directly under a fluorescence microscope to examine the surface vasculature. Thick vibratome sections (100 μm) are recommended to view vasculatures deeper than 100 μm from the tissue surface.

If perfusion fails with either tomato lectin or DiI, you can obtain useful information by transmitted light imaging, using hemoglobin absorption of the red blood cells, as your primary contrast. This is described in Subheading 3.5. The images will not be confocal optical sections, but you can still obtain useful information. We have successfully used RGB transmitted light mode Z-series tile scanning to acquire 200 μm thick Z-series from excised mouse skin.

When perfusing sacrificed mice, we encourage harvesting additional tissues and sharing them with colleagues interested in specific organs or tissues. You can contact your veterinarian about tissue sharing.

4 Results

Fluorescent labeled tomato lectin perfused blood vessels and DsRed2 fluorescent protein expressing tumor cells in hemisectioned mouse brains are readily visible by eye with a 10× objective lens and appropriate optics. The orthotopically implanted U87MG-DsRed2 human glioblastoma cell line tumors were typically imaged when 1–4 mm diameter. Foreknowledge of size was based on MicroMRI, Xenogen in vivo imaging data, and/or growth curves of the tumor model. Fluorescein-tomato lectin perfused blood vessels appear neon green to the eye when viewed through a Leica I3 (longpass green fluorescence) filter set; the DsRed2 tumor mass is opaque orange and the surrounding brain tissue is weakly green fluorescent. With the N2.1 filter set, DsRed2 tumors are bright orange-red, Texas Red-streptavidin–biotin–tomato lectin
appears red (and is used with nonfluorescent tumors); Alexa Fluor®
647-streptavidin–biotin-tomato lectin vessels are crimson-red, normal
brain tissue is dimmer orange. For tissue sections we often stain
nuclei with both DAPI and To-Pro-3 since our confocal micro-
scope does not have a UV laser for DAPI excitation; the former
fluorophore is blue-white with the Leica A UV excitation/visible
longpass emission filter set while To-Pro-3 is crimson-red with the
N2.1 set and can be imaged by the confocal optics with the 568
and/or 633 nm laser lines; likewise, LysoTracker Red can be imaged
with either the I3 or N2.1 filter sets and with 568 or 488 nm laser
excitation. In the future, there may be value in perfusing vessels
with both fluorescent-tomato lectin and cell permeable counter-
stains such as DAPI or Hoechst 33342/33258, SYTO dyes,
LysoTracker, etc.

Choice of laser line(s), laser power(s), confocal beamsplitter
(i.e., RSP500 vs. TD488/568/633), pinhole size, detector
settings, and Z-step size, and Z-range (Z-series depth range) need
to be optimized for each specimen type. If necessary, sequential
scanning, i.e., with the Ar488 laser line with RSP500 beamsplitter
(or RSP525, which often works better for green dyes), followed by
HeNe633 with TD488/561/633 for NIR imaging, can be used.
Sequential scanning may optimize fluorophore excitation and
and collection of emission photons but trades off by requiring longer
exposure times. On the SP1 this is somewhat mitigated by keeping
the spectral detection band passes the same, using PMT1 for short
wavelength and PMT2 or 3 for long wavelength. With our visible
light confocal microscope, fluorescent tomato lectin and DsRed2
tumor cell masses, freshly excised mouse brains that had been per-
fused to flush most of the red blood cells, could be imaged to a
depth of between 100 and 150 μm. Other organs, i.e., liver, spleen,
kidney, could be usefully imaged to a maximum depth of 60–100 μm.
Two confocal microscope datasets of ex vivo brain tumor cells
orthotopically implanted in a mouse brain are included with the
book CD, along with the Leica LCSLite confocal software image
viewer. The datasets were consecutive Z-series of 258 and 178 μm
with 1 mm × 1 mm field of view. The Leica SP1/DMIRBE settings
were: pinhole 1.0, Argon ion 488 nm laser line was used (12 o’clock
laser power knob, 69 % AOTF), RSP500 reflection shortpass
dichroic beamsplitter, PMT gains ~780, PMT1 wavelength range
502–537 nm, and PMT2 range 579–710 nm. The Leica LCS con-
focal acquisition software (not the free LCSLite software) includes
cytofluorogram and spectral deconvolution processing options.
We opted not to use these, but instead to use a relatively narrow
PMT1 virtual band pass of 35 nm to acquire the fluorescein-tomato
lectin images, and a well separated PMT2 band pass of 130 nm for
the DsRed2 fluorescence.

The accompanying DVD has a confocal dataset and the free
Leica LCS Lite visualization software. The g60m07 top 01 data is
a 258 μm Z-series, with maximum projections of fluorescein tomato lectin of blood vessels (Fig. 1) and DsRed2 fluorescence of human U87MG glioblastoma cells expressing hrLuc-RFP-TK tribrid fusion proteins (Fig. 2). Color overlays of maximum, average and triple view orthogonal projections, plus the entire Z series is on the CD. The CD also includes a movie (AVI format) of each channel, plus color overlay of the Z-series (false colored for better visualization) and a 360 rotating view. For comparison with classic histology, a Pathscan histology slide scan is also on the CD.

DiI perfusion of the lungs is describe in Methods (Subheading 3.9) and is adapted from [13]. DiI (lowest purity Sigma-Aldrich product) is less expensive than fluorescent tomato lectin. DiI is a member of a family of lipophilic carbocyanine fluorophores that are available in other excitation and/or emission colors: DiA, DiO, DiI, DiD, and DiR. The Di_ family have relatively broad excitation and emission spectra compared to many immunofluorescence fluorophores, e.g., Alexa Fluor 488 or Cy3, or to quantum dots, such as eFluor 605NC (eBiosciences, licensed from Evident Technologies) or QD625 (Invitrogen/Molecular Probes). The Di_ family is also characterized by different lipid lengths, which result in preferential localization to plasma membrane or mitochondria and endoplasmic reticulum. At University of Miami,
we now use DiI nearly exclusively for blood vessel painting. We have not (yet) done so, but expect to be able to combine DiI blood vessel painting with immunofluorescence by using digitonin as a tissue permeabilizing agent—instead of Triton X-100 or saponin—based on the results of Matsubayashi et al. [43].

Fluorescent lectin or DiI endothelial cell labeling can also be combined with DNA counterstaining, either in vivo [44] or by immersing the tissue in a DNA binding dye solution ex vivo, as briefly described in Subheading 3.4.

If a mouse fails to perfuse, you can use the absorption contrast of hemoglobin in red blood cells to acquire single field of view or tile scan single images or Z-series. The images will not be confocal, but you can still resolve multiple focal planes. The tissue needs to be transparent enough to get the light through (inquisitive users can also try reflection confocal mode). We illustrate this in supplemental data RGBZZZZ (if the editors want the data set or sets) where we used 458, 561, and 633 nm sequential scan tracks for blue, green, and red, respectively, for XY stage tile scanning with 200 μm Z-series (90 min scan). Because hemoglobin absorbs in both the blue and green, we could have decreased total scan time (to 60 min) by using 458 or 488 nm laser line for both the blue and green channel (cyan lookup table).
5 Discussion

Having been impressed by the success of R.M. Zucker in low-magnification confocal microscopy [7, 8], we decided to evaluate tumor microvessels labeling by 10× magnification confocal microscopy. Prior to the current method with unfixed hemi-sectioned brain tissue, we had previously established that we could image by confocal microscopy over 800 μm deep into hemi-sectioned mouse brains by using a bright red fluorescent latex vascular cast (S. Yamada and authors). However, the disadvantages of the vascular cast method, including 1 week tissue clearing time, fragility of cleared tissue, potential loss of antigenicity, and concern as to whether the latex penetrated all blood vessels before hardening, led us to rapid fluorescent-tomato lectin imaging in unfixed tissue. In unfixed mouse brain, we can image fluorescent cells and structures from the hemi-sectioned surface to 80–150 μm deep in DsRed2 expressing tumor masses and >150 μm deep in normal brain tissue. With Alexa Fluor® 647-streptavidin–biotinylated tomato lectin we have >200 μm depth is not unusual, though it is unnecessary for our project. The greater depth afforded by Alexa Fluor® 647 vs. fluorescein is more likely due to the increased tissue penetration of far red 647 nm (or 568 nm) excitation and near infra-red (650–800 nm) fluorescence emission, because longer wavelength light scatters less and is typically absorbed less, rather than the number of fluorophores per lectin molecule for the dye–streptavidin–lectin compared to dye–lectin.

Debbage et al. [10–12] evaluated many different fluorescent lectins. They obtained excellent results with several, though oddly did not evaluate tomato lectin conjugates. Microscopic blood vessel imaging has been achieved with many probes and techniques, including classic histology, immunohistochemistry, fluorescence conventional, confocal and multi-photon microscopies (see Introduction—a full review is beyond the scope of this article). One of the simplest—and has the virtue of being applicable to human specimens—is to simply immerse a formalin fixed biopsy or tissue block in dilute eosin, which results in bright fluorescent red blood cells and vessel structures in high magnification 100 μm thick confocal microscope data sets [45]. The importance of labeling and visualizing endothelial cells in context was highlighted by Chi et al. [46], who found, by mRNA expression microarray analysis, tremendous diversity of endothelial cells from microvasculature, macrovasculature and by organ and tissue.

Multimodal imaging synergies of longitudinal in vivo bioluminescent, fluorescent, and radiographic (MicroMRI, MicroCT, Volumetric CT, MicroSPECT, MicroPET, plain film X-ray) results in more information per subject than conventional sacrifice at each time point pathology methods. With longitudinal cancer therapy
studies, small numbers of mice can be selected at well chosen time points, labeled intravitally with blood vessel markers, such as the fluorescent tomato lectin used here, sacrificed, and the organs of interest imaged post vivo. The organs can then be examined further by conventional histology, immunohistochemistry, immunofluorescence, and in situ hybridization. Quantitation of tumor blood vessels using radiographic or light microscopic datasets has not been standardized. Pathologists and tumor biologists have shown great interest in histology quantitation of tumor blood vessels, but there is no consensus method [47]. Confocal fluorescence microscopes can image to depths of 100 μm, but are less expensive and more widely available to researchers than are multi-photon excitation microscopes which have maximal depth penetration of 500–1,000 μm [3, 48]. Hillman and Moore [49] invented a method, dynamic contrast enhancement (DyCE) to use differences in blood perfusion rates to segment organs and tumors in live mice, to assist in identifying the anatomical location(s) of molecular imaging agents such as near infrared fluorophore conjugated antibodies or firefly luciferase (DyCE is now available at http://www.cri-inc.com/products/dyce.asp). Simon [50] reported that what is now called selective plane illumination microscopy (SPIM) can be used to acquire single focus plane or an extended focus image using lateral sheet illumination. See ref. 51 for update on current uses of SPIM.

We anticipate further improvements as luciferase substrates are optimized for in vivo imaging (Promega’s EnduRen™ for Renilla luciferase), transgenic mice with specific cells expression of fluorescent proteins [6, 52], fluorescent proteins whose photophysics are optimized for deep tissue microscopy (i.e., long wavelength fruity mRFP1 derivatives [30, 53]), and luciferase-fluorescent protein fusion proteins are optimized for both bioluminescent resonance energy transfer and emission from animals and deep tissues [20, 27, 28]. These improvements will be useful for both macroscopic imaging, i.e., using the Xenogen IVIS instrument with hrLuc-optimized red fluorescent protein fusions to maximize BRET in vivo imaging, and the optimized red fluorescent protein fusion for confocal microscopy. We are especially looking forward to a high efficiency BRET fusion of effLuc [15] with an mRNA (lack of) secondary structure and codon optimized version of mKate2 [54]. We also note that Rabinovich et al. [15] have developed improved Gaussia luciferase and Renilla luciferase, that may serve as useful BRET donors for fluorescent proteins in vitro and in vivo, for example replacing the RLuc8 donor in mOrange-RLuc8 [55] with erLuc8 (Rabinovich pers comm.), and fully optimizing mOrange.

Fluorescent tomato lectin labeled blood vessels serve as excellent landmarks for confocal/multiphoton imaging of thick tissue. These are complemented by labeling specific cell populations with fluorescent proteins and labeling all nuclei with Hoechst 33342 or
other DNA staining dyes. The simplicity of our approach involves minimal fixation and processing of the mouse brain for imaging, and is compatible with further processing of the tissue for histology, immunohistochemistry and in situ hybridization.

Since moving to University of Miami, we have switched to the less expensive DiI perfusion method described briefly here [13, 36]. We have obtained excellent confocal Z-series with both many tissues, including tumor masses, heart and trachea (M. Jawad, pers comm.), retina (Y.L. and R.W.), muscle and skin (M. Jawad, pers. comm., J.B. and K.W.), and brain [36]. DiI has broad absorption and emission spectra, so you will see DiI blood vessels with standard GFP filter sets when searching by eye. The DiI bleedthrough into the GFP confocal fluorescence channel can be reduced by careful selection of emission filter (e.g., BP505-530 instead of BP505-550 or LP505) or using a spectral confocal microscope such as Leica SP1, SP2, or SP5 or Zeiss LSM710 (GFP and DiI are often bright enough that even a Zeiss LSM510META detector can separate these two fluorophores).

DiI has broad excitation and emission spectra. DiI at standard [13] concentration is much brighter than EGFP+ cells. This is both bad and good. Bad because DiI is excitable at 488 nm and has some fluorescence in the standard EGFP green emission channel of 500–530 nm. Good because it is unnecessary to use multitrack mode when imaging EGFP and DiI: use 488 nm excitation and two emission channels, such as 500–530 nm for EGFP and 550–650 nm for DiI. Crosstalk of DiI into the green channel can be corrected by subtraction (ex. EGFP = “green” − [“red”/scaler], where scaler is a scaling factor), or by spectral unmixing software in the acquisition or analysis software. Note that for quantitative correction the user needs to avoid detector saturation. The software may also need to account for different detector gain settings for the green and red channels (PMTs are intrinsically linear with respect to photons in to data out—for any single gain setting).

For deep tissue imaging of blood vessels DiI also excites well by multiphoton excitation fluorescence microscopy.

DiI blood vessel labeling should be combined with recent huge improvements in luciferase technology [15–18], that go beyond what Gambhir and colleagues had previously achieved. Rabinovich et al. [15] have generated improved firefly, Renilla and Gaussia luciferases (eFFLuc, eRLuc8, eGLuc). Mezzanotte et al. [16] reported on thermostable firefly luciferase (the same labs have also worked on improving the related click beetle luciferases). Saito et al. [17] fused Venus yellow fluorescence protein to RLuc8 to produce efficient bioluminescence energy transfer (BRET). Nagai et al. [18] have improved Venus–RLuc8 even further, taking advantage of efficient BRET to improve the quantum yield per coelenterazine substrate oxidation from 0.05 for RLuc8 to 0.70 for their SuperStar fusion construct. Neither Mezzanotte nor Saito and
Nagai et al. have taken advantage of optimizations of Rabinovich et al. [15], suggesting that further improvements are possible. An additional benefit of fusing Venus to RLuc8 is that this construct includes a fluorescent protein for in vivo and ex vivo imaging confocal and multiphoton excitation fluorescence microscopy. Venus YFP is also compatible with STED fluorescence nanoscopy (http://www.mpibpc.mpg.de/groups/hell/STED_Dyes.html) should SuperStar be fused to a specific protein or organelle targeting sequence.

DiI, as well as other in vivo blood vessel labeling methods (fluorescent tomato lectin, dextran) is compatible with new “living window” models—including real time in vivo immunophenotyping [22], to facilitate maximizing quantitative data from longitudinal in vivo studies of tumor growth and angiogenesis.

Acknowledgments

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We are grateful to Sam Gambhir for providing the tribrid hrLuc-DsRed2-TK reporter gene construct prior to publication (Ray et al. [20]). We thank Denise Petersen, Karen Pepper, and Don Kohn, CHLA Gene Vector Core, for inserting the tribrid reporter gene into the lentivirus vector and transducing U87MG cells. We thank Dr. Ignacio Gonzalez for histology slide preparation and tissue diagnoses. Our thanks to Ignacio Gonzalez, Dr. Rex Moats, Dr. Mike Rosol, Maya Otto-Duessel, and Dr. Shawn Chen, for discussions.
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The University of Miami Leica SP5 and MP-ND4/SP5/FCS/FLIM confocal microscopes were purchased with funds from the Diabetes Research Institute Foundation. Rong Wen and Yiwen Li are supported by the National Eye Institute and Bascom Palmer Eye Institute.

Appendix

Instrumentation

Few publications provide complete specifications on the instrumentation used. Each confocal microscope is built from specialty parts, many of whose performance vary between units. It is unlikely that any two confocal microscopes perform identically on all tests—see Lerner and Zucker [40] for examples. It is unlikely that the same confocal microscope performs identically on the same tests performed on sequential days, weeks, months or years. The intensity of the laser lines change over time (seconds and hours) and intensity changes can be confounded by focus drift of the microscope stage and/or warping of the specimen. In this appendix we list many of the components present in the Leica SP1 confocal DMIRBE inverted microscope that was delivered to CHLA in March 2000 and since upgraded with several new components. We recognize that in 2013 the SP1 is a discontinued model, but think a detailed explanation will help the reader. A major difference between the SP1 and the newer SP2 and SP5 models is that the latter have an acousto-optical beam splitter (AOBS) that replaces the primary laser dichroic beamsplitter(s) for most visible light lasers (the 405 nm and multiphoton laser do not use the AOBS). A correctly calibrated AOBS should enable collection of fluorescence emission from as close as 5 nm of the laser line. The laser light rejection from the emission light path can be disabled for reflection mode imaging.

The Leica SP1 confocal microscope has three lasers, with the Krypton ion laser having been replaced by diode-pumped solid-state (DPSS) laser in November 2004 (Table 1). The photomultiplier tubes (PMTs) in the SP1 are integrated with individual spectrophotometer-style scanning slits (Calloway [38], Tauer and Hils [39]) and the performance depends on correct alignment of the assembly (Table 2). High resolution spectral scanning (5 nm slits, 1 nm
step size, 200 steps, i.e., from 450 to 650 nm) of either the tungsten-halogen transmitted light source or laser lines reflected from a Nanofilm or Leica mirror slide, have revealed problems at different times with PMT assemblies (fixed with a service visit).

For a core facility where many different specimens are imaged, i.e., fluorescein-tomato lectin blood vessels and DsRed2 fluorescent

<table>
<thead>
<tr>
<th>Laser type</th>
<th>Manufacturer</th>
<th>Model</th>
<th>Wave-lengths</th>
<th>Power (mW)</th>
<th>Maximum power (10× lens output)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arion ion (Ar)</td>
<td>Uniphase</td>
<td>Ar-2211-65MLQYV</td>
<td>457</td>
<td>65</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>476</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>488</td>
<td>2.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>514</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Krypton ion (Kr)</td>
<td>Melles Griot</td>
<td>Omnichrome series 43, model 643R-LICA-B02</td>
<td>568</td>
<td>20</td>
<td>2.14</td>
</tr>
<tr>
<td>Diode-pumped solid-state microchip (DPSS)</td>
<td>Melles Griot</td>
<td>85 YCA 010</td>
<td>561</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Helium-Neon (HeNe)</td>
<td>Uniphase</td>
<td>1300 series</td>
<td>633</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Uniphase is now JDS Uniphase (http://www.jdsu.com). Power is the manufacturer’s rating (laser head output, all laser lines). Maximum power is measured at the microscope specimen at full power with the TD488/568/633 beamsplitter, through the 10×/0.30 dry lens, 10× zoom, Coherent power meter model FM part #33-0506 with “visible detector” (Leica field service engineer kit). The Ar and Kr lasers are only operated at full power (4 o’clock) for this test—the laser knob is set to the 12 o’clock position for standard use. The DPSS and HeNe use on/off switch so their power is only controlled with the SP1 AOTF “neutral density” attenuation control. The original Kr laser and power supply (installed 3/2000) died and was replaced under service contract (12/2001) and retired (11/2004) in favor of the DPSS 561 nm laser.

<table>
<thead>
<tr>
<th>PMT</th>
<th>Model</th>
<th>Type/grade</th>
<th>Remarks</th>
<th>Range (nm)</th>
<th>Peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R6358</td>
<td>LMA/UV</td>
<td>Low dark current</td>
<td>185–830</td>
<td>530</td>
</tr>
<tr>
<td>2</td>
<td>R6357</td>
<td>MA/UV high</td>
<td>High sensitivity</td>
<td>185–900</td>
<td>450</td>
</tr>
<tr>
<td>3</td>
<td>R6357</td>
<td>MA/UV high</td>
<td>High sensitivity</td>
<td>185–900</td>
<td>450</td>
</tr>
<tr>
<td>Trans</td>
<td>R6350</td>
<td>Sb-Cs/UV</td>
<td>UV to visible, general purpose</td>
<td>185–650</td>
<td>340</td>
</tr>
</tbody>
</table>

PMT1, 2, and 3 are assemblies of a photomultiplier tube integrated with virtual band-pass sliders of the SP1 scanhead confocal spectrophotometer. The detection efficiency depends on the PMT detector and the double slider slit being aligned correctly. PMT photocathode quantum efficiency curves were published by Zipfel et al. [56]. For simultaneous imaging of fluorescein and DsRed2, PMT1 is ch00 and either PMT2 or PMT3 is ch01. Choice of second PMT is made on the basis of whether the PMT2 assembly (tube plus virtual band-pass unit) is operating correctly (for example, in 12/2004, the unit produced readings of +40 nm for a Nanofilm reflection slide laser spectral scanning test. The assembly was replaced under service contract and performed acceptably).
protein tumor cell masses, CFP → YFP FRET, and Cy5.5-RGD peptide labeled cells, having a large selection of filter sets on hand for viewing specimens by eye is crucial (Table 3). Compared to the price of the confocal microscope (~$360,000) and annual service contract (~$17,000), filter sets at <$1,000 each are inexpensive compared to the entire system. Our filter sets are shared between the Leica SP1 confocal DMIRBE microscope (four cube positions, three filter cubes used plus one empty position for confocal scanning) and a Leica DMRXA/RF8 microscope with eight filter cubes. To maximize compatibility, our Leica MZFLIII motorized fluorescence stereomicroscope has many matched filter sets (the MZFLIII uses a plastic slider with one exciter and two emission filters, and a mirror to reflect Leica Xe 75 W light to the specimen).

The confocal scanhead uses an acousto-optical tunable filter (AOTF) as a wavelength selective neutral density control. The AOTF gives much finer and reproducible control over laser power than do the knobs on the Ar and Kr lasers (the DPSS and HeNe lasers do not have knobs, only on/off switches). The AOTF enables adjusting laser power independently for each of the six laser lines in ~0.4 % steps, from 0 to 100 %. The actual output depends on the laser power knob. The Ar457 line power fluctuates over time, at any knob setting, and tends to be low at low knob power settings.

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The Leica SP1 scanhead has five beamsplitters (Table 4) for directing the laser light from the AOTF neutral density unit to the objective lens and specimen, and then back from the objective lens to the prism spectral dispersion element and PMT slits/tube assemblies. Our SP1 has a triple dichroic, TD488/568/633, three reflection shortpass (RSP465, RSP500, RSP525) and one reflection/transmission (RT30/70) beamsplitters. The numbers indicate appropriate laser lines (TD filter), approximate 50 % reflection wavelength (RSPs), or approximate reflection/transmission performance (RT). The choice of 30 % reflection (laser light to the specimen) and 70 % transmission (specimen reflection and/or fluorescence emission) is a trade-off of wanting to excite the specimen with as much light as possible, but even more importantly, collecting as much (in focus) light as possible. If the SP1 had much more powerful lasers, it might make more sense to use a RT10/90 (resulting in 0.1 × 0.9 = 0.09 total throughput, but crucially, 90 % of the emitted light), than our RT30/70 (0.3 × 0.7 = 0.21 total throughput, 70 % of the emitted light) (Table 4). The scanhead beamsplitter numbers do not tell the whole story. For any given laser line, fluorophore(s), specimen (autofluorescence), and PMT assembly spectral band pass (especially if out of whack), a particular confocal beamsplitter may be found empirically to outperform another. In particular, we sometimes find the 488 nm laser line and RSP525 beamsplitter often outperforms the RSP500 beamsplitter for fluorescein imaging. See Table 5 for objective lenses.
Table 3
Microscope filter sets for visual imaging

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Filter cube</th>
<th>Part number</th>
<th>Excitation</th>
<th>Dichroic</th>
<th>Emission</th>
<th>Typical fluorophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leica</td>
<td>A</td>
<td>513824</td>
<td>BP 340–380</td>
<td>400</td>
<td>LP425</td>
<td>DAPI</td>
</tr>
<tr>
<td>Leica</td>
<td>I3</td>
<td>513828</td>
<td>BP450–490</td>
<td>510</td>
<td>LP515</td>
<td>Fluorescein, EGFP</td>
</tr>
<tr>
<td>Leica</td>
<td>N2.1</td>
<td>513832</td>
<td>BP515–560</td>
<td>580</td>
<td>LP590</td>
<td>Cy3, DsRed</td>
</tr>
<tr>
<td>Chroma</td>
<td>HQ Cy5.5</td>
<td>41022</td>
<td>HQ665/45×</td>
<td>Q695LP</td>
<td>HQ725/50 m</td>
<td>Cy5.5, To-Pro-3</td>
</tr>
<tr>
<td>Chroma</td>
<td>HQ Cy3</td>
<td>41007a</td>
<td>HQ545/30×</td>
<td>Q570LP</td>
<td>HQ610/75 m</td>
<td>Cy3</td>
</tr>
<tr>
<td>Chroma</td>
<td>HQ Fluorescein</td>
<td>41001</td>
<td>HQ480/40×</td>
<td>Q505LP</td>
<td>HQ535/50 m</td>
<td>Fluorescein, EGFP</td>
</tr>
<tr>
<td>Chroma</td>
<td>DAPI</td>
<td>31000</td>
<td>D360/40×</td>
<td>400DCLP</td>
<td>D460/50 m</td>
<td>DAPI</td>
</tr>
<tr>
<td>Chroma</td>
<td>DAPI/Green/Red</td>
<td>61000v2</td>
<td></td>
<td></td>
<td></td>
<td>Triple</td>
</tr>
<tr>
<td>Chroma (ASI)</td>
<td>SKY</td>
<td>SKYv3</td>
<td></td>
<td></td>
<td></td>
<td>Green, orange, red, Cy5, Cy5.5</td>
</tr>
<tr>
<td>Chroma</td>
<td>CFP</td>
<td>31044v2</td>
<td>D436/20×</td>
<td>455DCLP</td>
<td>D480/40 m</td>
<td>Cyan Fluorescent Protein, auto-fluorescence (DAPI, fluorescein, Cy3 slides)</td>
</tr>
<tr>
<td>Chroma</td>
<td>YFP</td>
<td>41028</td>
<td>HQ500/20×</td>
<td>Q515LP</td>
<td>HQ535/30 m</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>Chroma</td>
<td>CFP→YFP FRET</td>
<td>31052</td>
<td>D436/20×</td>
<td>455DCLP</td>
<td>D535/30 m</td>
<td>Cyan→Yellow FP FRET</td>
</tr>
<tr>
<td>Chroma</td>
<td>Rex</td>
<td>Custom</td>
<td>E650SP</td>
<td>700DCXR</td>
<td>RG715</td>
<td>Cy7</td>
</tr>
<tr>
<td>Chroma</td>
<td>Qdot/Evitag LPES</td>
<td>Custom</td>
<td>E460spuvV2</td>
<td>475DCXRU</td>
<td>HQ480LP (w/AR)</td>
<td>QDots, Evitags</td>
</tr>
<tr>
<td>Chroma</td>
<td>Custom IGS</td>
<td>Custom</td>
<td>E550spuv+21003Pol</td>
<td>20/80 beamsplitter (Chroma 21008)</td>
<td>E450LP+21003Pol</td>
<td>Immunogold staining, DIC analyzer, polarized fluorescence</td>
</tr>
</tbody>
</table>

Data from Leica Microsystems and Chroma Technology product literature. See Ploem [58] and Reichman [59] for information on filter cubes and filters, respectively. The above cubes are interchangeable between the Leica SP1 confocal DMIRBE and Leica DMRXA/RF-8 microscopes. The CHLA Image Core has identical filter sets, for most of the above sets, for a Leica MZFLIII stereomicroscope (excitation and emission filter slider). Confocal imaging is performed with no filter set in the microscope. Non-confocal fluorescence images can be acquired using the epi-illumination arc lamp and any of the filter sets with wide open detection pinhole. This is an inefficient method because spot scanning is used with wide-field illumination, but is useful for localizing DAPI stained nuclei on a visible-lasers only system. Transmitted light RGB images can be acquired with sequential imaging of the 457 nm (blue), 568 nm (yellow-green), and 633 nm (red) lasers with the RT30/70 scanhead beamsplitter and the transmitted light PMT detector. Spectral data for most of the filters and dyes discussed can be found at http://works.bepress.com/gmcnamara/9/ [57]
### Table 4
**Scanhead beamsplitters**

<table>
<thead>
<tr>
<th>Scanhead beamsplitter</th>
<th>Excitation laser line(s)</th>
<th>Fluorophore(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD488/568/633</td>
<td>488, 568, 633</td>
<td>488: Fluorescein, Alexa Fluor 488, EGFP; 568: Cy3, DsRed; 633: To-Pro-3, Alexa Fluor 647, Cy5, Cy5.5</td>
</tr>
<tr>
<td>RSP465</td>
<td>457</td>
<td>CFP, CFP → YFP FRET, SYTO40, SYTO45</td>
</tr>
<tr>
<td>RSP500</td>
<td>488 (457, 476)</td>
<td>Fluorescein, Alexa Fluor 488, EGFP</td>
</tr>
<tr>
<td>RSP525</td>
<td>514 (457, 476, 488)</td>
<td>Fluorescein, Alexa Fluor 488, EGFP, YFP</td>
</tr>
<tr>
<td>RT30/70</td>
<td>Any</td>
<td>Any</td>
</tr>
</tbody>
</table>

*Note: The confocal microscope can also be operated in reflection mode, with the PMT assembly band pass set to match the laser line, i.e., 486–490 nm band pass for the 488 nm Ar laser line. All of the dichroic filters can reflect some laser light and transmit the laser light and some (a little or a lot) of any emission wavelength. Spending a few moments to evaluate all combinations is advised. Published fluorescent dye spectra (e.g., [http://www.spectra.arizona.edu/](http://www.spectra.arizona.edu/) and [http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Labeling-Chemistry/Fluorescence-SpectraViewer.html](http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Labeling-Chemistry/Fluorescence-SpectraViewer.html)) are useful but not definitive.*

### Table 5
**Confocal microscope objective lenses**

<table>
<thead>
<tr>
<th>Leica lens</th>
<th>10×/0.30</th>
<th>20×/0.40</th>
<th>40×/1.25–0.7</th>
<th>63×</th>
<th>10×/0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markings</td>
<td>HC</td>
<td>N</td>
<td>HCX</td>
<td>1.4 NA</td>
<td>HC Pl Apo</td>
</tr>
<tr>
<td></td>
<td>Plan</td>
<td>Plan</td>
<td>Corr</td>
<td>Plan Apo</td>
<td></td>
</tr>
<tr>
<td>Immersion</td>
<td>Dry</td>
<td>Dry</td>
<td>Oil</td>
<td>Oil</td>
<td>IMM (water, glycerol, oil)</td>
</tr>
</tbody>
</table>

Condenser lens: 0.9 NA, with condenser numerical aperture control and field aperture. Transmitted path has two filters above the condenser. One is a neutral density filter. The other is a polarizer. The polarizer enables Nomarski differential interference contrast (DIC) imaging both by eye (lamp to this polarizer to Wollaston prism in condenser to specimen to A/C/E Wollaston prism in microscope, to polarizer slider in base) and by confocal (opposite light path to the above, with the exception that the polarizer in the base is left out of the light path, since the laser light is polarized)

In 2005 one of us (G.M.) moved to City of Hope National Medical Center

### Web Sites

<table>
<thead>
<tr>
<th>Web Site</th>
<th>URL</th>
</tr>
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<tbody>
<tr>
<td>Chroma Technology Corp</td>
<td><a href="http://www.chroma.com">http://www.chroma.com</a></td>
</tr>
<tr>
<td>Mattek Corp.</td>
<td><a href="http://www.mattek.com">http://www.mattek.com</a> and <a href="http://www.glass-bottom-dishes.com">http://www.glass-bottom-dishes.com</a></td>
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</table>

(continued)
<table>
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<tr>
<th>CD Image Files</th>
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<tr>
<td>LCSLite200871.exe</td>
</tr>
<tr>
<td>LCSLite2051347a.exe</td>
</tr>
<tr>
<td>LCSLite2611537.exe</td>
</tr>
</tbody>
</table>

*Note*: All versions of LCS Lite require Administrator privileges to install on a Windows PC. LCS Lite is the free, limited capabilities version of the LCS (Leica Confocal Software) used for acquisition. Leica confocal download sit is ftp://ftp.llt.de/softlib. LCS Lite is available for download from ftp://ftp.llt.de/softlib/LCSLite/ (2.6.1, dated 12/09/2004 is final version). Leica LAS AF Lite is available at ftp://ftp.llt.de/softlib/LAS_AF_Lite/ (version 2.1.0 is dated 5/29/2009).
References


