Abstract: Three-dimensional fluorescence laminar optical tomography (FLOT) can achieve resolutions of 100-200 µm and penetration depths of 2-3 mm. FLOT has been used in tissue engineering, neuroscience, as well as oncology. The limited dynamic range of the charge-coupled device-based system makes it difficult to image fluorescent samples with a large concentration difference, limits its penetration depth, and diminishes the quantitative accuracy of 3D reconstruction data. Here, incorporating the high-dynamic-range (HDR) method widely used in digital cameras, we present HDR-FLOT, increasing penetration depth and improving the ability to image fluorescent samples with a large concentration difference. The method was tested using an agar phantom and a B6 mouse for brain imaging in vivo.

OCIS codes: (170.3880) Medical and biological imaging; (170.3010) Image reconstruction techniques; (170.6960) Tomography; (170.2520) Fluorescence microscopy.

References and links
1. E. M. C. Hillman, O. Bernus, E. Pease, M. B. Bouchard, and A. Pertsov, “Depth-resolved optical imaging of transmural electrical propagation in perfused heart,” Opt. Express 15(26), 17827–17841 (2007).
2. E. M. C. Hillman, D. A. Bous, A. M. Dale, and A. K. Dunn, “Laminar optical tomography: demonstration of millimeter-scale depth-resolved imaging in turbid media,” Opt. Lett. 29(14), 1650–1652 (2004).
3. Q. Tang, J. Wang, A. Frank, J. Lin, Z. Li, C. W. Chen, L. Jin, T. Wu, B. D. Greenwald, H. Mashimo, and Y. Chen, “Depth-resolved imaging of colon tumor using optical coherence tomography and fluorescence laminar optical tomography,” Biomed. Opt. Express 7(12), 5218–5232 (2016).
4. A. Dunn and D. Boas, “Transport-based image reconstruction in turbid media with small source-detector separations,” Opt. Lett. 25(24), 1777–1779 (2000).
5. B. Yuan, S. A. Burgess, A. Irmanalboob, M. B. Bouchard, N. Lehrer, C. Bordier, and E. M. C. Hillman, “A system for high-resolution depth-resolved optical imaging of fluorescence and absorption contrast,” Rev. Sci. Instrum. 80(4), 043706 (2009).
6. Q. Tang, V. Tsytasarev, A. Frank, Y. Wu, C. W. Chen, R. S. Erzumulu, and Y. Chen, “In Vivo Mesoscopic Voltage-Sensitive Dye Imaging of Brain Activation,” Sci. Rep. 6(1), 25269 (2016).
7. S. Yuan, Q. Li, J. Jiang, A. Cable, and Y. Chen, “Three-dimensional coregistered optical coherence tomography and line-scanning fluorescence laminar optical tomography,” Opt. Lett. 34(11), 1615–1617 (2009).
8. E. M. C. Hillman, A. Devor, M. B. Bouchard, A. K. Dunn, G. W. Krauss, J. Skoch, B. J. Bacskai, A. M. Dale, and D. A. Boas, “Depth-resolved optical imaging and microscopy of vascular compartment dynamics during somatosensory stimulation,” Neuroimage 35(1), 89–104 (2007).
9. S. A. Burgess, D. Ratner, B. R. Chen, and E. M. Hillman, “Fiber-optic and articulating arm implementations of laminar optical tomography for clinical applications,” Biomed. Opt. Express 1(3), 780–790 (2010).
10. S. Björn, V. Ntzia iristhos, and R. Schulz, “Mesoscopic epifluorescence tomography: reconstruction of superficial and deep fluorescence in highly-scattering media,” Opt. Express 18(8), 8422–8429 (2010).
11. L. Zhao, V. K. Lee, S. S. Yoo, G. Dai, and X. Intes, “The integration of 3-D cell printing and mesoscopic fluorescence molecular tomography of vascular constructs within thick hydrogel scaffolds,” Biomaterials 33(21), 5325–5332 (2012).
12. M. S. Ozturk, V. K. Lee, L. Zhao, G. Dai, and X. Intes, “Mesoscopic fluorescence molecular tomography of reporter genes in bioprinted thick tissue,” J. Biomed. Opt. 18(10), 100501 (2013).
13. M. S. Ozturk, D. Rohrbach, U. Sunar, and X. Intes, “Mesoscopic fluorescence tomography of a photosensitizer (HPPH) 3D biodistribution in skin cancer,” Acad. Radiol. 21(2), 271–280 (2014).

14. S. Björn, K. H. Englemier, V. Ntziachristos, and R. Schulz, “Reconstruction of fluorescence distribution hidden in biological tissue using mesoscopic epifluorescence tomography,” J. Biomed. Opt. 16(4), 046005 (2011).

15. Y. Chen, S. Yuan, J. Wierwille, R. Naphas, Q. A. Li, T. R. Blackwell, P. T. Winnard, V. Raman, and K. Glunde, “Integrated Optical Coherence Tomography (OCT) and Fluorescence Laminar Optical Tomography (FLOT),” IEEE J. Sel. Top. Quant. 16(4), 755–766 (2010).

16. M. S. Ozturk, C. W. Chen, R. Ji, L. Zhao, B. N. B. Nguyen, J. P. Fisher, Y. Chen, and X. Intes, “Mesoscopic Fluorescence Molecular Tomography for Evaluating Engineered Tissues,” Ann. Biomed. Eng. 44(3), 667–679 (2016).

17. Q. Tang, J. Lin, V. Tsytsarev, R. S. Erzurumlu, Y. Liu, and Y. Chen, “Review of mesoscopic optical tomography for depth-resolved imaging of hemodynamic changes and neural activities,” Neurophotonics 4(1), 011009 (2017).

18. M. A. Robertson, S. Borman, and R. L. Stevenson, “Estimation-theoretic approach to dynamic range enhancement using multiple exposures,” J. Electron. Imaging 12(2), 219–228 (2003).

19. B. C. Madden, “Extended intensity range imaging,” Technical Report, GRASP Laboratory, University of Pennsylvania (1993).

20. P. E. Debevec and J. Malik, “Recovering high dynamic range radiance maps from photographs,” in ACM SIGGRAPH 2008 classes (ACM, 2008), p. 31.

21. L. Lian, Y. Deng, W. Xie, G. Xu, X. Yang, Z. Zhang, and Q. Luo, “High-dynamic-range fluorescence molecular tomography for imaging of fluorescent targets with large concentration differences,” Opt. Express 24(17), 19920–19933 (2016).

22. P. Fei, Z. Yu, X. Wang, P. J. Lu, Y. Fu, Z. He, J. Xiong, and Y. Huang, “High dynamic range optical projection tomography (HDR-OPT),” Opt. Express 20(8), 8824–8836 (2012).

23. C. Vinegoni, C. Leon Swisher, P. Fumene Feruglio, R. J. Giedt, D. L. Roussos, S. Stapleton, and R. Weissleder, “Real-time high dynamic range laser scanning microscopy,” Nat. Commun. 7, 11077 (2016).

24. C.-W. Chen, A. B. Yeatts, E. E. Coates, J. P. Fisher, and Y. Chen, “Experimental Demonstration of Angled Fluorescent Laminar Optical Tomography for Tissue Engineering,” in Biomedical Optics and 3-D Imaging (Optical Society of America, Miami, Florida, 2012), p. BTuA4.5.

25. C. W. Chen and Y. Chen, “Optimization Of Design Parameters for Fluorescence Laminar Optical Tomography,” J. Innov. Opt. Health Sci. 4(03), 309–323 (2011).

26. S. Yuan, C. A. Roney, J. Wierwille, C. W. Chen, B. Xu, G. Griffiths, J. Jiang, H. Ma, A. Cable, R. M. Summers, and Y. Chen, “Co-registered optical coherence tomography and fluorescence molecular tomography for simultaneous morphological and molecular imaging,” Phys. Med. Biol. 55(1), 191–206 (2010).

27. C. P. Liang, J. Wierwille, T. Moreira, G. Schwartzbauer, M. S. Jafri, C. M. Tang, and Y. Chen, “A forward-imaging needle-type OCT probe for image guided stereotactic procedures,” Opt. Express 19(27), 26283–26294 (2011).

28. Q. Tang, C.-P. Liang, K. Wu, A. Sandler, and Y. Chen, “Real-time epidural anesthesia guidance using optical coherence tomography needle probe,” Quant. Imaging Med. Surg. 5(1), 118–124 (2015).

29. L. Wang and S. L. Jacques, “Use Of a Laser Beam with an Oblique Angle of Incidence to Measure the Reduced Scattering Coefficient of a Turbid Medium,” Appl. Opt. 34(13), 2362–2366 (1995).

30. M. Johns, C. Giller, D. German, and H. Liu, “Determination of reduced scattering coefficient of biological tissue from a needle-like probe,” Opt. Express 13(13), 4828–4842 (2005).

31. J. L. Sandell and T. C. Zhu, “A review of in-vivo optical properties of human tissues and its impact on PDT,” J. Biophotonics 4(11-12), 773–787 (2011).

32. Q. Tang, Y. Tsytsarev, C. W. Chen, B. Xu, G. Griffiths, J. Jiang, H. Ma, A. Cable, R. M. Summers, and Y. Chen, “In Vivo Voltage-Sensitive Dye Imaging of Subcortical Brain Function,” Sci. Rep. 5, 17325 (2015).

33. V. Tsytsarev, K. Premachandra, D. Takeshita, and S. Bahar, “Imaging cortical electrical stimulation in vivo: fast intrinsic optical signal versus voltage-sensitive dyes,” Opt. Lett. 33(9), 1032–1034 (2008).

34. V. Tsytsarev, D. Pope, E. Pumbo, A. Yablonskii, and M. Hofmann, “Study of the cortical representation of whisker directional deflection using voltage-sensitive dye optical imaging,” Neuroimage 53(1), 233–238 (2010).

35. V. Tsytsarev, C. Bernardelli, and I. Maslov, “Living Brain Optical Imaging: Technology, Methods and Applications,” J. Neurosci. Neuroeng. 1(2), 180–192 (2012).

36. J. Huisken, J. Swoger, F. Del Bene, J. Wittbrodt, and E. H. K. Stelzer, “Optical sectioning deep inside live embryos by selective plane illumination microscopy,” Science 305(5686), 1007–1009 (2004).

37. J. P. Culver, V. Ntziachristos, M. J. Holboke, and A. G. Yodh, “Optimization of optode arrangements for diffuse optical tomography: A singular-value analysis,” Opt. Lett. 26(10), 701–703 (2001).

38. P. C. Hansen and D. P. O’Leary, “The use of the L-curve in the regularization of discrete ill-posed problems,” SIAM J. Sci. Comput. 14(6), 1487–1503 (1993).

39. X. Intes, J. Ripoll, Y. Chen, S. Nioka, A. G. Yodh, and B. Chance, “In vivo continuous-wave optical breast imaging enhanced with Indocyanine Green,” Med. Phys. 30(6), 1039–1047 (2003).

40. T. J. Muldoon, S. A. Burgess, B. R. Chen, D. Ratner, and E. M. C. Hillman, “Analysis of skin lesions using laminar optical tomography,” Biomed. Opt. Express 3(7), 1701–1712 (2012).

41. N. Ouakli, E. Guevara, S. Dubeau, E. Beaumont, and F. Lesage, “Laminar optical tomography of the hemodynamic response in the lumbar spinal cord of rats,” Opt. Express 18(10), 10068–10077 (2010).
1. Introduction

Laminar optical tomography (LOT) is a promising optical imaging method that collects scattered light travelling through different depths by using multiple detectors, and reconstructs the depth-resolved images through mathematical deconvolution [1–3]. LOT can achieve a resolution of 100-200 µm with 2-3 mm penetration depths [1, 2, 4–7]. It was initially utilized to image hemodynamic changes based on absorption contrast [2, 8, 9]. Soon after, LOT was rapidly adapted for fluorescent molecular imaging, which was termed either fluorscence laminar optical tomography (FLOT) [1, 5–7] or mesoscopic fluorescence molecular tomography (MFMT) [10, 11], and several improvements or alternative system designs were investigated [5, 6, 10–14]. FLOT has been reported to quantify depth-resolved distribution of fluorescence-labeled tumors [13, 15, 16], to image fluorescence-labeled cells in tissue engineering [11, 12, 16], and to visualize neural activities in mouse brains in vivo [6, 17]. In the FLOT system configuration, a charge-coupled device (CCD) or electron-multiplying CCD (EMCCD) can be used as the array detector since either one has a higher sampling density in comparison to a photomultiplier tube (PMT) array or an avalanche photodiode (APD) array [6, 16]. During signal collection in FLOT with reflectance imaging mode, the photons collected at larger source-detector separations have a higher statistical probability of travelling through deeper tissues. In general, the detector with a larger source-detector separation collects a lower signal compared to a detector closer to the illumination source, indicating that the deeper area will have a low signal-to-noise ratio (SNR), which will limit reconstruction accuracy [16]. We can increase either the excitation power or exposure time to enhance the collected signals from deeper regions. However, all pixels acquired from CCD/EMCCD have the same gain or exposure time, meaning that increasing the excitation power or exposure time will saturate the pixels near the illumination source very quickly. Another scenario is when imaging fluorescent samples with a large concentration difference (e.g., inhomogeneous dye loading, tumors at different stages), the area with higher fluorescence concentrations will easily become saturated, while areas with a low fluorescence concentration will have a low SNR, which may affect the quantitative accuracy of FLOT. Ultimately, the insufficient dynamic range of the CCD/EMCCD limits the penetration depth and quantitative accuracy of FLOT.

A high-dynamic range (HDR) method based on a multiple-exposure scheme is widely used in digital cameras and smartphones [18–20]. Taking advantage of the multiple-exposure-based HDR method, HDR optical projection tomography (HDR-OPT), HDR laser-scanning microscopy (HDR-LSM), and HDR fluorescence molecular tomography (HDR-FMT) were recently reported [21–23]. Localization of fluorescent targets with a large concentration difference is effectively improved with HDR-FMT. Good quantitative accuracy were demonstrated on both phantom and in vivo animal experiments [21].

In this paper, we present an HDR-FLOT method to increase both its dynamic range and penetration depth. To assess the potential of this method, we first fabricated an agar phantom in which three 150-µm capillaries filled with different concentrations of Cy 5.5 solution were inserted at similar depths. Then, we obliquely inserted one capillary within the brain of a mouse in vivo to illustrate the improved penetration depth of HDR-FLOT for brain imaging. Our data demonstrated the feasibility of HDR-FLOT in increasing the dynamic range and penetration ability of FLOT, and provide a potentially improved mesoscopic tomography method for imaging neuronal activities.

2. Methods

2.1 Construction of 2D HDR fluorescence images

The basic idea of HDR-FLOT is to implement 3D reconstruction in FLOT based on 2D HDR fluorescence images [20, 21]. The first step to reconstruct 2D HDR images is to recover the CCD response function. The exposure $X$ of a pixel can be defined as the product of irradiance
and the exposure time \( t \), where irradiance \( E \) is influenced by the optical properties of the sample as well as illumination from the light source. From the CCD, we can obtain a digital number \( Z \) (CCD output), which is related with \( X \) at every pixel. We can define this relationship as CCD response function \( f \). Since the background noise could cause deviation of the CCD response function, we first subtract the noise from the original CCD output, so that \( Z \) at every pixel will be:

\[
Z - Z_{\text{sample}} - Z_{\text{background}}
\]

(1)

\( Z_{\text{sample}} \) are the values of samples from CCD, and \( Z_{\text{background}} \) are background values in the corresponding images. Then, we can denote the response function as:

\[
Z_{ij} = f(E, t_j)
\]

(2)

where \( i \) is the index of pixels and \( j \) is the index of exposure time. Since \( f \) is monotonic, we can rewrite Eq. (2) as:

\[
f^{-1}(Z_{ij}) = E, t_j
\]

(3)

Taking the natural logarithm of both sides, we can obtain:

\[
\ln f^{-1}(Z_{ij}) = \ln E_i + \ln t_j
\]

(4)

Then, we can rewrite the equation with a defined function \( g = \ln f^{-1} \) as:

\[
g(Z_{ij}) = \ln E_i + \ln t_j
\]

(5)

The parameters \( E \) and function \( g \) are unknown, but there is a linear relationship between \( g(Z_{ij}) \) and \( \ln E_i \). We change the problem to solving the quadratic objective function as follows:

\[
O = \sum_{i=1}^{N} \sum_{j=1}^{T} \omega(Z)[g(Z_{ij}) - \ln E_i - \ln t_j]^2 + \sigma
\]

(6)

where \( \omega(Z) \) is a weighting function. When the signal is low or saturated, the pixel value \( Z \) and exposure \( X \) may fit the response function poorly. Thus, we may give smaller weighting to those pixels. Simply, we can define \( \omega(Z) \) as [20]:

\[
w(Z) = \begin{cases} 
Z - Z_{\text{min}} & \text{for } Z \leq \frac{1}{2}(Z_{\text{min}} + Z_{\text{max}}) \\
Z_{\text{max}} - Z & \text{for } Z > \frac{1}{2}(Z_{\text{min}} + Z_{\text{max}})
\end{cases}
\]

(7)

\( \sigma \) is introduced as a smoothness parameter to make sure that the function \( g \) is continuous and smooth [20]. The best estimate of CCD response function \( g \) will be obtained when \( O \) in Eq. (6) has its minimum value. The commonly used method to satisfy the equation is the least-square method for linear regression [20, 21].

Because of the limited memory of a computer, we pick 2500 pixels instead of all the 512*512 pixels for calculation (i.e., the sampling rate is 1/10). The best estimate of the CCD response function \( g \) will be obtained when \( O \) reaches its minimum value. After the CCD response function \( g \) has been obtained, we can calculate irradiance \( E \) by Eq. (5). The weighting function \( w \) is introduced again here to give a higher weighting value to data at
reliable ranges (the middle of the response function), but a lower value to the ranges near the extremes. Then, we can reconstruct the 2D HDR image as [20, 21]:

\[
\ln E^{HDR} = \frac{\sum_{i=1}^{N} \sum_{j=1}^{T} \omega(Z) g(Z_{ij}) - \ln t_j}{\sum_{i=1}^{N} \sum_{j=1}^{T} \omega(Z_{ij})}
\] (8)

After that, we can obtain the 2D HDR image using all the images with different exposure times, and a series of 2D HDR images can be obtained at each scanning position, which will later be used for 3D HDR-FLOT reconstruction.

2.2 System setup

The schematic of the FLOT system is shown in Fig. 1(A), which is similar to the system reported previously [6, 24]. A pigtailed 637-nm laser diode was used as the light source (LP637-SF70, Thorlabs Inc.). Light from the pigtailed laser diode was first collimated by an objective lens. A cylindrical lens was used to shape the collimated light into a line-field illumination with a full line-width at the half maximum of ~26 µm at the focal plane. An iris was utilized to control the length of the line illumination. The backscattered light and emitted fluorescent light were collected back through another objective lens, a filter wheel (695-nm long-pass emission filter for fluorescence imaging and no filter for recording reflectance images), and finally onto a 12-bit CCD camera (EM-CCD, Cooke) [6]. A pair of polarizers was used to reject the specular reflection from the sample surface. The illumination angle was set at 45°, rendering an ~30° transmission angle in tissue (n ~ 1.33) [6], as our previous studies indicated that angled illumination and detection configurations can improve both resolution and depth sensitivity [6, 25]. We set the focal plane slightly below the sample surface to compensate for the defocusing effect. The sample was scanned laterally in the scanning direction X (perpendicular to the line illumination direction Y) using a motorized stage.

To co-register the FLOT fluorescence information with morphological/anatomical information, we also imaged the samples with optical coherence tomography (OCT). The OCT system shown in Fig. 1(B) was described previously [7, 26–28]. A wavelength-swept laser centered at 1310 nm with a 100-nm bandwidth was used in this frequency-domain OCT system as a light source [7, 26, 27]. The wavelength-swept frequency was 16 kHz and the output power from the laser was approximately 16 mW. About 97% of the laser power was split 50:50 to the sample and the reference arms of a fiber-based Michelson interferometer [7, 26, 27]. A balanced detector (BD) was used to receive the interference fringes from different depths encoded with different frequencies. The remaining 3% of the laser output power was sent to a Mach-Zehnder interferometer (MZI) to generate a frequency-clock signal with a uniformly spaced optical frequency to trigger the sampling of the OCT signal [7, 26, 27]. Then, a fast Fourier transform of the interference fringes was performed to obtain the depth-resolved axial profile [7, 26, 27].

2.3 Experimental design

All animal experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and the protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park campus.

(1) Phantom study

To demonstrate the capability of HDR-FLOT to image fluorescent samples with a large concentration difference, an agar phantom was fabricated with three 150-μm glass capillaries (ID: 0.15 mm, OD: 0.25 mm, VitroCom Inc.) inserted at similar depths filled with different
Cy 5.5 concentrations. First, the three glass capillaries were filled with 2 µM, 1 µM, and 0.2 µM Cy5.5 solutions, respectively (Cyanine5.5 azide, Lumiprobe Corporation). The filled capillaries were then fixed side by side on the empty petri dish. Then, 1.5-g agar powder (Now Inc.), 0.3-mL 20% intralipid solution (Hospira Inc.), and 49-mL PBS buffer were mixed and stirred well. After heating in a microwave for ~2 minutes, the mixed liquid gel was poured into the petri dish on which the glass capillaries were fixed and the capillaries were submerged in the liquid gel. After the liquid gel cooled and solidified (~10 minutes), the gel was ready to be imaged. The scattering coefficient (µs) of the agar phantom was determined from the reflectance data using oblique-incidence spectroscopy to be ~2.3 mm$^{-1}$ (g = 0.9, n = 1.33, $\mu_a$ = 0.01/mm, $\mu'_s$ = 0.23/mm at 635 nm) [29]. We further fabricated another agar phantom with 1.5-g agar powder, 2.5-mL 20% intralipid solution, and 49-mL PBS buffer, providing a reduced scattering coefficient $\mu'_s$ = ~1.2/mm at 635 nm, which is within the range of most biological tissue [30, 31]. To co-register 3D OCT and FLOT images, a piece of black tape was stacked on the phantom surface near the glass capillaries, as shown in Fig. 1(C), as a fiducial marker.

(2) Animal study
LOT/FLOT has been applied in recording 3D neural activities and hemodynamic responses in the mouse brain in vivo [6, 8]. Increasing the penetration depth would broaden the application of FLOT for neuroscience research. Since the optical properties of the living mouse brain are too complicated to mimic using a simple phantom, a 150-µm glass capillary filled with voltage-sensitive dye (commonly used for reporting neural activities [6, 32–35]) was inserted into a mouse brain in vivo to demonstrate the feasibility of HDR-FLOT to increase penetration depth.

A 4-5 month-old B6 mouse was anesthetized with urethane (1.15 g/kg body weight). The mouse’s head was shaved before being placed into a stereotaxic frame (Stoelting Co.) [6, 32]. Then, a cranial window was made over the left parietal cortex (about 4 × 4 mm). The surface of the dura mater was cleaned with hemostatic sponges dipped in artificial cerebrospinal fluid (ACSF) as described previously [6, 32]. One 150-µm glass capillary filled with voltage-sensitive dye RH-1691 (Optical Imaging Ltd.; 1.0 mg/ml in ACSF) was then slowly inserted into the exposed area. The cortical surface was then covered with high-density silicone oil. The scattering coefficient ($\mu_s$) of the living mouse brain was determined from the reflectance data using oblique-incidence spectroscopy (g = 0.9, n = 1.33, $\mu_a$ = 0.01/mm, $\mu'_s$ = 0.82 /mm at 635 nm) [6, 29]. The brain phantom was then imaged by OCT and FLOT subsequently.

2.4 Data acquisition and image reconstruction
For FLOT image acquisition, the excitation laser line was first focused on the border (X = 0) of the sample field of view (FOV) [6]. At each scanning position, one 2D XY image (512x512, with pixel dimension of 30.8 µm) was obtained with different exposure times (e.g., 100 ms, 200 ms, 500 ms, 1000 ms, and 2000 ms). Next, the sample was moved by the motorized stage in the X direction with a step size of 61.6 µm to another illumination/collection position. Another 2D XY data set with different exposure times was obtained. This process was repeated until scanning was completed on the entire sample [6]. The raw measurement had the format of X Y T. A typical 3D data set recorded $512 \times 512 \times 500$ X Y T voxels for five different exposure times. To fit the experimental data to the theoretical model used for 3D reconstruction, the sample surface should be found [6]. To obtain the surface profile of the samples, the same FOV was scanned with a 100 ms exposure time at lower laser power (driver current: ~40 mA) to avoid saturating the CCD camera. The emission filter was removed by rotating the filter wheel during the acquisition of the reflectance images. Without the emission filter, most of the acquired signal was from the reflection of the illumination light at the sample surface. This signal would then be used to indicate the location of the sample-air interface. The raw measurement of reflectance data set
had the same format of XYT (X = 512, Y = 512, T = 100) [6]. The ROI was then imaged by the OCT system described above to obtain the 3D morphological tomography.

To reconstruct the FLOT tomograms, first, the fluorescence images with the same exposure time at different scanning positions were arranged as one group (e.g., the data set for all the images at different scanning positions with 100 ms exposure time: XYT₁₀₀ₘ₉, X = 512, Y = 512, T₁₀₀ₘ₉ = 100). The separated images were then applied to obtain the CCD response curve and to further construct the HDR images based on the protocol described in Section 2.1. After that, we had six data sets for each sample, five with different exposure times and one with a constructed HDR data set. Each 3D data set was then downsized to 256 × 256 × 100 voxels to match the pixel size in XYT dimensions. The raw measurements of the reflectance data were stacked together according to the geometrical relationship between the illumination plane and the detection FOV, as described previously [6, 36]. The stacked reflectance data were also used to co-register 3D FLOT images with the 3D OCT data based on the black tape for the agar phantom and the shape of the mouse brain’s blood vessels. Reconstructing the fluorescence images with different exposure times was via the same method as described previously [6]. We assumed a first-order Born approximation to obtain the linearity between the CCD measurement F (with background noise subtracted) and the fluorophore distribution C (i.e., for each FOVₓ₉, F = JC, with J the weight or sensitivity matrix) [6, 24]. J was constituted by Monte Carlo simulation and the reciprocity principle. J was later decomposed by singular value decomposition (SVD) [6, 37]. Last, the undetermined system was solved by least square fitting and Tikhonov regularization [6, 25]; the regularization parameter was determined by the L-curve criterion [38, 39]. In our experiment, for each FOVₓ₉, 100 source-detector pairs and 100 scanning positions starting from the surface were chosen to constitute F. Each reconstructed FOVₓ₉ was composed of 100 × 100 pixels with the same pixel size of ~61.6 µm. The 3D FLOT image (FOVₓᵧ₉) was constituted by juxtaposing individual FOVₓ₉ along the Y-direction. Six 3D FLOT images (five with different exposure times and one with a constructed HDR data set) were reconstructed to compare their performances.

2.5 Data statistical analysis

Data are expressed as mean ± standard deviation. Statistical analyses were carried out using MATLAB. A Student’s t-test was used to compare the response error ratio (RER), diameter difference ratio (DDR), and penetration depth obtained from different exposure times and HDR mode. Statistical significance is considered at \( p = 0.05 \).

3. Results and discussion

3.1 Co-registration of the 3D FLOT and OCT images

To obtain the distribution of the capillaries (e.g., depth and angle) in the agar phantom and living mouse brain, the samples were first imaged by the OCT imaging system. A piece of black tape was used as a landmark on the phantom surface near the glass capillaries [see Fig. 1(C)]. Figures 1(D) and (E) show the images of the same black tape from the 3D stacked reflectance LOT data set and the 3D OCT data set, respectively. Figure 1(F) presents the co-registered OCT and FLOT images using the black tape as a fiducial mark, which can guarantee that the structure provided by OCT is in the same ROI imaged by FLOT. Based on the OCT images, we can further evaluate the capability and accuracy of the HDR-FLOT system in the following studies.
3.2 Phantom study

Figure 2(A) shows a representative cross-sectional OCT image of the phantom with three capillaries filled with Cy 5.5 solution. The locations of the three capillaries with different Cy 5.5 concentrations are indicated by the 3 differently colored arrows (red: 2 µM, yellow: 1 µM, green: 0.2 µM). All three capillaries are at similar depths of ~650-750 µm. We denote them as C1 (2 µM), C2 (1 µM), and C3 (0.2 µM), respectively, in the following sections.

Figure 2(B) is the 3D rendered image of these three capillaries from OCT. Figures 2(C-E) show 2D images obtained from FLOT at one scanning position using exposure times of 100 ms, 500 ms, and 2000 ms, respectively. With an exposure time of 100 ms, C1 and C2 clearly stand out from the background, while C3 is difficult to distinguish. C3 appears when the exposure time is increased to 500 ms, while C1 starts to get saturated at this exposure time. Further increasing the exposure time to 2000 ms also increases the fluorescence intensity of C3 to the middle of the CCD signal range, while C1 and C2 are both saturated, which means we cannot recover the quantitative relationship of the fluorophore concentrations in these capillaries. Figure 2(F) shows the recovered CCD response curve obtained by using all of the images with five different exposure times. As the exposure time increases, the data points shift to the high range of the CCD and some points become saturated, indicating the limited dynamic range of the CCD system. The recovered CCD response curve and all the images with five different exposure times were then used to construct the 2D HDR fluorescence image, shown in Fig. 2(G). In the 2D HDR fluorescence image, the dynamic range of the fluorescence projection image is effectively improved. To assess the accuracy of the 2D HDR fluorescence images, the fluorescence value was averaged from a 5x5 region of interest (ROI)
Fig. 2. (A) Image from OCT system of the three capillaries filled with Cy 5.5 solution, indicated by the 3 arrows (red: 2 µM (C1), yellow: 1 µM (C2), green: 0.2 µM (C3)). (B) 3D rendered images of the 3 capillaries from the OCT system. (C-E) 2D images obtained from FLOT using exposure times of 100 ms, 500 ms, and 2000 ms. (F) Recovered CCD response curve; the solid black line is the fitted curve (x axis is in loge scale). (G) Constructed 2D HDR-FLOT image.

Fig. 3. Average fluorescence intensity of the raw 2D fluorescence images as a function of true Cy 5.5 concentration with exposure times of 100 ms (A), 500 ms (B), 2000 ms (C), and the HDR method (D). The fluorescence values were normalized to the maximum value (n = 100). (E) Average CCD response error ratios with different exposure times (n = 100).
at 100 capillary positions for each capillary. The averaged fluorescence values were then normalized and plotted as a function of true Cy 5.5 concentration, as shown in Fig. 3. (We normalized the CCD values at 2 µM as 1, then normalized the CCD values at 1 µM and 0.2 µM to the CCD values at 2 µM.) The cyan dashed line depicts the ideal linear relationship of the normalized fluorescence values as a function of true Cy 5.5. With an exposure time of 100 ms, the fluorescence values from C1 and C2 perform well in linearity, while C3 is off the line and has a low quantitative accuracy, since the signal in this area is weak and has a low SNR [Fig. 3(A)]. Since the fluorescence signal from C1 has already been overexposed for data obtained with exposure times of 500 ms and 2000 ms, the linearity relationship is completely distorted and the fluorescence signals are unable to quantify the true Cy 5.5 concentration [Fig. 3(B) and (C)]. In comparison, the fluorescence signals from the constructed 2D HDR images show an excellent linear response to the true Cy 5.5 concentration [Fig. 3(D)].

To quantify the accuracy of normalized CCD values with different exposure times from the ideal linear relationship, the measured response error ratio (RER) was defined as following: $RER = \left| \frac{NCV - IV_p}{IV_p} \right| \times 100\%$, where $NCV$ represents the normalized CCD values from measurements and $IV_p$ represent the ideal values at different Cy 5.5 concentrations (since we normalized the CCD values at 2 µM as 1, we just need to calculate $RER$ for $p = 1 \mu M$ & $0.2 \mu M$ with $IV_{p=1,0.2,M} = 0.5, IV_{p=0.2,0.5} = 0.1$ ). We averaged the $RER$ at each scanning position and further averaged all the $RER$ from all the 100 scanning positions. 2D HDR images present significantly smaller DDR within 15% of that from all the other exposure times [Fig. 3(E)].

Figures 4(A-C) show the 3D FLOT reconstructed images with exposure times of 100 ms, 500 ms, and 2000 ms. As expected, C3 cannot stand out from the background with 100 ms exposure time [Fig. 4(A)]. Although increasing the exposure time will enhance the SNR of this capillary, the shapes of C2 and C3 become distorted because overexposure and noise become more apparent, as shown in Fig. 4(C). With 2D HDR fluorescence images, the 3D reconstructed image can resolve all three capillaries with different concentrations of Cy 5.5 simultaneously, as shown in Fig. 4(D). To quantify the fluorescence intensity of the reconstructed FLOT 3D images, the normalized average fluorescence intensities of the three capillaries were again plotted as a function of true Cy 5.5 concentrations, as shown in Fig. 5. Similar to the 2D data, the 3D FLOT images reconstructed with 2D HDR fluorescence...
images show the smallest RER within 20%. To further assess the size accuracy of capillaries from the reconstructed 3D FLOT images with different exposure times, the 3D FLOT images were then co-registered with the corresponding 3D OCT images, as shown in Fig. 4(E-H). All the three capillaries from OCT data were labelled green for better visualization. The sizes of capillaries reconstructed from FLOT were evaluated as the diameter difference ratio (DDR) as follows: \( DDR = \left| \frac{D_{\text{FLOT}} - D_{\text{OCT}}}{D_{\text{OCT}}} \right| \times 100\% \), where \( D_{\text{FLOT}} \) is the diameter of the capillary measured from FLOT, and \( D_{\text{OCT}} \) is the diameter of the capillary measured from OCT, respectively. We first analyzed the DDR for FLOT with different exposure times at different Cy 5.5 concentrations, as shown in Fig. 5(F). HDR-FLOT and 2000 ms exposure time provide similar DDR which is better than those from 100 ms and 500 ms at the lowest Cy 5.5 concentration (0.2 µM). At the modest Cy 5.5 concentration (1 µM), the best DDR comes from 500 ms, while HDR-FLOT also presents relatively good DDR. When it goes to high Cy 5.5 concentration (2 µM), 100 ms offers the best DDR with HDR-FLOT the second-best. Then we define the average DDR was the mean of the three DDRs calculated from the three capillaries. As shown in Fig. 5(G), the averaged DDR is around 46% with an exposure time of 100 ms and C3 cannot be resolved (\( D_{\text{FLOT}} = 0 \) since C3 cannot be resolved at this exposure time). When the exposure time increases, averaged DDR increases, indicating the sizes measured from FLOT deviate from the correct values. HDR-FLOT presents significantly better average DDR (within 40%) than any of the other three cases and with all the three capillaries clearly displayed.

Fig. 5. Average fluorescence intensity of the 3D images as a function of true Cy 5.5 concentration with exposure time 100 ms (A), 500 ms (B), 2000 ms (C), and the HDR method (D). The fluorescence values were normalized to the maximum value (\( n = 50 \)). (E) Average fluorescence response error ratios with different exposure times (\( n = 50 \)). (F) Diameter difference ratios for FLOT with different exposure time at different Cy 5.5 concentrations compared to OCT data (\( n = 50 \)). (G) Average diameter difference ratios for FLOT with different exposure times compared to OCT data (\( n = 5 \)).

In another phantom with a reduced scattering coefficient of ~1.2 mm\(^{-1}\), similar to the previous case, only parts of C3 can stand out from the background with 100 ms and 500 ms exposure, as shown in Figs. 6(A) and (B). Although increasing the exposure time will enhance the SNR of this capillary, the shapes of C1 and C2 become distorted because overexposure and noise become more obvious for 2000 ms [Fig. 6(C)]. With 2D HDR fluorescence images, the 3D reconstructed image can resolve all three capillaries with different concentrations of Cy 5.5 simultaneously [Fig. 6(D)]. Because of the increased
scattering, there are more noises in the reconstructed 3D images and the constructed capillaries appear larger in diameter (more distortion).

![Fig. 6. 3D FLOT reconstructed images in the agar phantom with $\mu_s' = 1.2/mm$ at 635 nm. (A-C) 3D FLOT reconstructed images with exposure times of 100 ms, 500 ms, and 2000 ms. (D) 3D HDR-FLOT reconstructed image. (E-H) Co-registered 3D FLOT reconstructed images with corresponding OCT images (green). The Max in the colormap is the maximum value in the image and Min represents the value: (Background + (Max-Background)/2).]

### 3.3 Oblique glass capillary in living mouse brain

Figure 7(A) shows the 150-µm capillary in a mouse brain in vivo. The upper row in Fig. 7(B) shows the 3D FLOT-reconstructed images with exposure times of 100 ms, 500 ms, 2000 ms, and HDR-mode. The reconstructed image using 500 ms exposure time has a clear increase in depth than that using 100 ms exposure time, as a longer exposure time will enhance the signals at deeper regions that have a lower SNR. On the other hand, we can also notice that there is more noise at shallower areas using an exposure time of 500 ms, as a higher exposure time will saturate the sample and increase the noise in the shallow areas. The saturation and noise become more obvious when using an exposure time of 2000 ms, resulting in the shape of the capillary being hardly distinguishable at the shallow areas. However, as indicated by the red arrow in the image, the capillary in the deeper area is displayed clearly, indicating that a higher exposure time overexposes the detectors closer to the illumination source, while enhancing the signals collected by the detectors farther away from the illumination source. In comparison, the capillary reconstructed by HDR-FLOT has a relatively deep penetration with a relatively low noise level at the shallow areas. The bottom row of Fig. 7(B) shows the reconstructed 3D FLOT images superimposed with the 3D OCT images (labelled in green). We define the reconstructed depth as the vertical length of the obliquely inserted capillary in the mouse brain under the preset threshold, which indicates the limitation of the depth that FLOT can reconstruct in the mouse brain. Figure 7(C) shows the quantitative penetration depth using different exposure times. With an exposure time of 100 ms, the reconstructed depth of the capillary can reach around 550 µm and up to around 800 µm with an exposure time of 500 ms. Our HDR-FLOT method presents a ~1 mm depth, more than 20% deeper than that at 500 ms. Although the reconstructed depth of the capillary reaches deepest (1.2 mm) with an exposure time of 2000 ms, it is not practical since the 3D shape of the capillary is distorted too much at the shallower regions. We further evaluated the DDR as a function of depth for each of the exposure times and the HDR image in Fig. 7(D). In the shallow range (within 400 µm), 100 ms provides the best DDR. HDR and 500 ms exposure time give similar DDVs while HDR has a deeper penetration depth. Further increasing the exposure time to 2000 ms results in a better DDR at deeper range (>700 µm) and larger penetration.
depth, while the signal cannot be distinguished from noises in the shallow range because of saturation.

![Fig. 7](image)

Fig. 7. (A) Photo of a 150-µm capillary in the mouse brain *in vivo*. (B) 3D FLOT reconstructed oblique capillary with exposure times of 100 ms, 500 ms, and 2000 ms, and HDR-FLOT. (C) Capillary depths in mouse brain from FLOT with exposure times of 100 ms, 500 ms, and 2000 ms and HDR-FLOT (n = 5). (D) Diameter difference ratios for FLOT with different exposure times as a function of depth compared to OCT data. The Max in the colormap is the maximum value in the image and Min represents the value: (Background + (Max-Background)/2).

4. Conclusion

In this paper, by combining the commonly used multi-exposure-based HDR method, we presented HDR-FLOT. We first imaged a phantom with three 150-µm capillaries filled with different concentrations of Cy 5.5 solution to demonstrate the feasibility of HDR-FLOT in imaging samples with large concentration differences. Then, we imaged a mouse brain *in vivo* with an oblique capillary inserted to show the improved penetration depth of HDR-FLOT for brain imaging. Our data indicate that HDR-FLOT has increased the detection dynamic range, allowing it to resolve samples with large concentration differences simultaneously in the same ROI, and the reconstructed 3D data are more quantitatively accurate. HDR-FLOT can also increase imaging depth by more than 20% compared to conventional FLOT in a mouse brain *in vivo*.

Depth-resolved LOT/FLOT has been demonstrated in imaging tumors/fluorescence-labeled tumors [13, 15, 16, 40], perfused rat hearts [1], and fluorescence-labeled cells in tissue engineering [11, 12], as well as for visualizing neural activities in mouse brain and spinal cord [6, 8, 41]. In a realistic experiment, usually we do not know the fluorescence situation/distribution of the sample, which means that it is difficult to choose a proper excitation power or exposure time for the unknown inhomogeneous fluorescence distribution. By employing the HDR-FLOT, samples with large concentration differences can be resolved simultaneously in the same ROI, and in a more quantitatively accurate way. Since light attenuation is exponential in biological tissues, while SNR gain with integration time grows with the square root of the integration time, HDR-FLOT may provide only limited improvement in penetration depth. However, in biological studies, small increases in penetration depth will add confidence to the result and may provide more information. For example, with more than a 20% depth increase compared to conventional FLOT in a mouse brain *in vivo*, we may be able to image the entire six layers in the mouse cortex with HDR-
FLOT compared to four layers. Hemodynamic response usually occurs in seconds; therefore, the LOT system with ~40 frames/s imaging speed can be used to study hemodynamic responses [8, 17]. Use of exogenous fluorescent dyes and transgenic animals can also aid in studying functional parameters, such as changes in membrane potential [voltage-sensitive dyes (VSD)] or ion concentrations (pH-, calcium-, chloride-, or potassium-sensitive dyes) [17, 35]. The time-resolved acquisition protocol to record the fast neural dynamics, which requires the biological response to be repeatable for each stimulation trial [17], was investigated for FLOT [1, 6, 17]. The HDR-FLOT presented in this paper can be incorporated with a time-resolved acquisition protocol to provide a potentially improved mesoscopic imaging method to image neuronal activities for functional brain mapping. In future, we can shorten each exposure time by increasing the excitation power and reducing the quantity of the exposure times, using two or three exposure times instead of the five, as used in this paper, to accelerate overall data acquisition time.

**Funding**

National Institutes of Health (NIH) (R21EB012215-01A1, R01EB014946-01A1).

**Acknowledgment**

We thank Kevin Cho from the University of Maryland for his helpful discussion.