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Development of a fluorescent microsphere technique for rapid histological determination of cerebral blood flow

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Abstract

The purpose of this study was to develop a more efficient fluorescent microsphere method to facilitate the rapid use of the histological technique and to enable its use in large tissue regions. Using fluorescent plate/slide imaging technology and automated detection and analysis software, we were able to rapidly image, detect, and count 3 separate microsphere colors in 200 μ m-thick tissue sections from piglet brain. In resting newborn piglets ($n = 6$) on isoflurane anesthesia, we measured a median total cerebral blood flow (CBF) of 105 ml/min/100g (range 27–206 ml/min/100g). Compared with other FM analysis methods, our method reduces the time required to determine blood flow, improves accuracy in lipid-rich tissues and large tissue regions and, unlike the radiolabeled microsphere method, can be combined with histological analysis.

Keywords

cerebral blood flow; fluorescent microspheres; pig

1. Introduction

The microsphere technique, using either radiolabeled or fluorescently labeled microspheres, has been developed as one of the reference standards for measuring regional tissue blood flow (Heymann et al., 1977; Prinzen and Glenny, 1994). While radiolabeled microspheres require minimal tissue and signal processing, differences in tissue sectioning from those used for histology and the health risks from radioactivity exposure limit their utility for coupling blood flow with histological measurements. Fluorescent microspheres do not have the same health hazards, but most quantitative methods require tissue digestion (Glenny et al., 1993; Powers et al., 1999), which again eliminates the opportunity to compare blood

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flow with histology in the same tissue sample. Furthermore, use of the tissue digestion technique on brain tissue of large animals is particularly problematic. The large white matter content increases the amount of lipid material, which is difficult to break down (Powers et al., 1999) and may result in variable amounts of tissue digestion and variable microsphere capture.

More recently, a histological fluorescent microsphere method has been developed that allows preservation of tissue for histopathological assessment (De Visscher et al., 2003; Luchtel et al., 1998). This method involves cutting tissue into 100 μm -thick histological slices, imaging the microspheres using epifluorescent microscopy, and manual counting of spheres. While this method only takes 3 hours of tissue processing per brain in the rat (De Visscher et al., 2006), it becomes impractical in large animal models where the region of interest sizes and number of microspheres counted increase dramatically. In the piglet brain, for example, the area of a typical coronal hemisection slice occupies 3–4 cm^2 , compared with 0.5–0.6 cm^2 in a rat brain, and a 3mm-thick section generates 30 such 100 μm slices. In our experience, this corresponds to 4–5 hours of tissue processing for just one microsphere color in one brain region. When the processing time is multiplied by the number of timepoints and regions of interest in large brains, this traditional microsphere counting technique becomes laborious and unrealistically time-consuming.

We present here a novel adaptation of a previously established fluorescent microsphere method (De Visscher et al., 2003) and demonstrate its utility by measuring cerebral blood flow (CBF) in piglets. We have improved the efficiency of this technique by using fluorescent plate/slide imaging technology that can image an entire slide at once coupled with a novel automated detection software algorithm. Our method reduces the amount of time required to determine blood flow by 80–90%, with a greater added time-savings for larger numbers of timepoints and regions, improves measurement accuracy in lipid-rich tissues as well as large tissue regions, and is easily combined with histological analysis.

2. Results

Imaging in each of the 3 fluorescent excitation-emission channels produces 3 separate gray-scale images (Figure 1a) for each tissue slice. The brightness-radius distribution of particles demonstrates a clear region of high-intensity FM pixels for each image, which is delineated by a polygon (Figure 1d). The automated program counts the particles within the polygon, and good correspondence between visible particles and counted particles is visualized by overlaying the counts onto the original image (Figure 1b). Occasionally, FMs in retained meningeal tissue are present on the slide which do not lie within the brain parenchyma and should not be included in CBF calculations. These few FMs (<2% of total counts) are easily visualized in this image overlay (Figure 1b, center), and counts are manually corrected. An overlay of the 3 resulting images as 3 different colors demonstrates clear separation of the FMs in each channel (Figure 1c).

The physiologic conditions did not vary significantly among the three colored FM injections (Table 2). We measured resting cerebral blood flow at three separate times in these piglets under 1 MAC isoflurane anesthesia (Figure 2). We found no right-to-left sided differences in the cerebrum by paired t-test ($p=0.15$) and no significant differences based on color of FM injection ($p=0.65$), or between animals receiving crimson FMs and those receiving red FMs ($p=0.68$) by nonparametric analysis. Median CBF (ml/min/100g, range) differed significantly by brain region ($p<0.003$), and was calculated as 105 ml/min/100g (27–206) in the cerebrum, 186 ml/min/100g (38–388) in the midbrain-pons, 235 ml/min/100g (72–622) in the medulla, and 166 ml/min/100g (36–346) in the cerebellum, over all colors in all animals.

Table 3 lists the FM counts of representative tissue slices from randomly selected animals imaged face up and face down in each of the 3 channels. None of the face up and corresponding face down counts differed by more than 10%, demonstrating good tissue penetration of the fluorescent signal through 200µm thick tissue.

3. Discussion

We have extended the histological technique developed by De Visscher et al. (De Visscher et al., 2003; De Visscher et al., 2006) to facilitate multiple CBF measurements in large tissue regions. The use of fluorescent slide imaging technology that can capture an entire slide at once in <5 minutes significantly reduces the acquisition time of the fluorescent microsphere images to only 1–2 hours per brain region. The depth of field of this device enables imaging of 200 µm thick slices with nearly all FMs in focus, yielding a tight cluster of FM sizes and intensities. By increasing the thickness of tissue sections from 100 to 200 µm, we were able to reduce both tissue cutting and tissue imaging times by 50%. Finally, the automated counting program shrinks the counting time to mere seconds per brain region or blood filter. This is in contrast to the significantly more laborious processes of tissue digestion and spectrophotometry, or fluorescent microscopy with manual counting which can take up to 2–3 days for a single similar measurement. The improved efficiency of our technique makes it particularly useful for large animal studies.

The absolute CBF results measured in neonatal piglets using this novel FM method are consistent with values reported in the literature. CBF values in the cerebrum range from 66–108 ml/min/100g in newborn piglets 1–6 days old under various anesthetic conditions (Buckley, 1986). Our values are in the upper end of this range, likely due to the use of isoflurane anesthesia, as inhaled anesthetics have been shown to increase basal CBF (Moore et al., 1994). Moore et al. reported forebrain CBF values in untreated controls of 97–127 ml/100g/min in 8–10 week piglets under isoflurane (Moore et al., 1994). Similarly, newborn piglets on a mixture of halothane and nitrous oxide had total CBF values of 95 ± 8 ml/min/100g (Leffler et al., 1989).

We were able to get good separation of 3 FM colors in the 3 optical filter channels of the fluorescent plate reader using optical bandwidths (Table 2) that are fairly broad relative to those typically used in spectrophotometry (Glenny et al., 1993; Walter et al., 1997), but narrower than those typical in epifluorescent microscopy (De Visscher et al., 2006). For colors in which bleed-through into multiple channels did occur, we were able to correct for this without significant loss of accuracy. While our particular filter sets limited the number of colors and, therefore, time points we were able to measure, the use of even narrower bandwidth filters would significantly decrease bleed-through and allow the inclusion of more colors.

An important consideration when using this technique is that imaging at a tissue's autofluorescence wavelength requires a careful balance between maximizing the signal-to-noise ratio and minimizing bleedthrough into other channels. For instance, brain tissue autofluorescence is greatest in the visible blue range, and decreases as the excitation wavelength increases. However, the blue-green microspheres (Molecular Probes) whose signal lies within this narrow range and does not bleed through into the adjacent yellow-green channel had insufficient signal to discriminate FMs from the background tissue autofluorescence. For other tissue types, the range and magnitude of auto-fluorescence should be assessed prior to FM color choice. In addition, to minimize the effect of autofluorescence across all wavelengths, one should use the minimum exposure necessary to visualize each FM color. In the case of brain tissue, for example, yellow-green and orange FMs were sufficiently bright to illuminate a radius of 3–5 pixels with 8ms exposure duration. Longer duration

exposures increased tissue background noise, decreasing signal-to-noise and accuracy of counts. Red and crimson FMs required longer exposure times (100 ms) in the Cy5HF channel to illuminate 3–5 pixels, but tissue autofluorescence was minimal in this channel and did not interfere with signal. It is important to note that tissue auto-fluorescence also increases over time after tissue cutting, as the sample gradually dries. Based on our experience, brain tissue auto-fluorescent signal generally peaks by 4–6 months after tissue cutting, particularly in the FITC or shorter wavelength range; thus, we recommend prompt tissue imaging.

Another limitation of this method is that measurement accuracy decreases when two different colors of FMs are injected at the same time. Although we have demonstrated good separation of different colors in tissue, clear separation is difficult in the mandatory reference blood withdrawal samples. The optical properties of the FMs against the backdrop of the filters used to capture the spheres from the reference blood samples broadened the emission bandwidth of the spheres. Therefore, there was an increase in the bleed-through of fluorescent signal to adjacent optical filters. This technical problem does not occur when imaging FMs in tissue. Consequently, two different FM colors cannot be injected simultaneously, and care must be taken to keep individual reference withdrawal blood samples separated from each other during processing.

Previous studies have determined that there should be at least 400 FMs per region of interest in order to minimize measurement error to <10% when using microspheres (Buckberg et al., 1971). We found that injecting as few as 1×10^6 FMs still resulted in >2000 FMs/3mm coronal tissue section in a single cerebral hemisphere under our particular experimental conditions. These numbers may be ideal for studying conditions which increase blood flow, or when spatial resolution is less important. Care must be taken, however, when significantly reducing the number of FMs injected or when studying very small regions or severely ischemic conditions, as the number of FMs in a tissue section may be reduced below 400, which would increase measurement error. This error is correctable by increasing region size or number of microspheres injected.

It is important to note that with this technique, microspheres must be spatially separated by at least one FM diameter in order to be counted as individual particles. This constraint puts an upper limit on the number of microspheres that can be injected at a single time-point. For instance, using $>4 \times 10^6$ FMs/injection under our particular experimental conditions dramatically increased the density of FMs in the tissue, resulting in 10–15% of FMs spaced closer than the resolution of our optical system, which we considered an unacceptably high error rate. This error is strictly flow-dependent and will only be reduced under lower flow conditions or by decreasing the number of FMs injected; it cannot be reduced by decreasing the size of the tissue sample. Therefore, the upper limit will depend on the particular experimental conditions, and it should be determined for a given model system before using this technique. When the appropriate number of FMs is injected with adequate mixing, closely spaced microspheres are a rare (<1%) event. In our model, we chose 2×10^6 FMs/injection, because it is close enough to the upper limit to optimize spatial resolution while allowing room for both dynamic increases and decreases in blood flow without increasing either high or low count errors.

Another issue with using $\geq 2 \times 10^6$ FMs/injection is that during filtration of reference withdrawal microspheres, a smaller percentage of FMs are retrieved on the first 2 filtration passes than with 1×10^6 FMs/injection, such that more than 3 filtrations are needed to confirm >95% recovery of FMs from the blood sample. We recommend using a 4th filtration step with a smaller pore filter (8µm pore size) when injecting $\geq 2 \times 10^6$ FMs at a withdrawal rate of 1 ml/min.

Finally, the thickness of tissue sections plays an important role in both technique efficiency and image quality. While 200 μm thick slices can be imaged with good focus, larger tissue thicknesses greatly increase the number of blurred FMs that appear as larger, dimmer objects and are more difficult to separate from background tissue noise (data not shown). However, one limitation with using a tissue thickness of 200 μm is the difficulty in cutting consistently even sections across large tissue areas. This difficulty increases dramatically with increasing tissue thicknesses $>200\text{ }\mu\text{m}$. We found that homogeneity of the agarose gel, a fresh sharp blade, and a steep (30°) blade angle are critical to maintaining uniform thickness of cuts, particularly in soft tissue such as brain. By decreasing blade advance speed and vibration frequency, we were able to improve uniformity and decrease the risk of tissue-gel separation. Even with these adjustments, the last 10–20% of embedded tissue adhered less well to the gel and tended to come out of the gel during cutting. We found that decreasing the thickness of tissue slices at this point increased tissue recovery from our sections. We stepped down tissue thickness to 150 μm then 100 μm for the last 4–5 slices in each tissue section. While using only 100 μm thick sections significantly reduces problems with uneven slicing, it doubles both the cutting and imaging times and is not recommended.

In conclusion, we have developed a novel fluorescent microsphere signal acquisition and analysis technique that can measure cerebral blood flow in large tissue regions efficiently. This technique significantly reduces the time to acquire and analyze fluorescent images by scanning an entire slide at once and accommodating up to 200 μm -thick tissue sections. While our technique is limited by the number of colors and the density of microspheres in the tissue that can be separated optically, these limits are well within the range of most research applications.

4. Materials and Methods

4.1. Normal piglet surgical preparation

Animal procedures were conducted in accordance with the principles of laboratory animal care established by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Six anesthetized 3–5 day old female piglets were used for this study. Each animal was induced with 4% isoflurane and intubated. Catheters were placed in both femoral arteries, one for mean arterial blood pressure (MAP) monitoring and one for reference microsphere blood withdrawals. In addition, one catheter was placed in a femoral vein for normal saline infusion (4cc/kg/hr maintenance fluids), and one in the left ventricle of the heart via the right carotid artery for fluorescent microsphere injections. Vital physiological parameters including MAP, heart rate, arterial oxygen saturation, end-tidal CO_2 , and body temperature were monitored and recorded every 15 minutes for the duration of each study. Animals were mechanically ventilated with room air and supplemental oxygen as needed to maintain normoxia and normocarbida. Isoflurane was reduced to 4.25–4.75% maintenance levels for the remainder of the study.

4.2. Microsphere injections

Three fluorescent microsphere (FM) injections were performed in each animal, using three different colors of 15 μm -diameter microspheres in random order: yellow-green, orange, and either red or crimson (Table 1, Molecular Probes, Invitrogen, CA, USA), with an interval of at least 1 hour between each time point. At each time point, $1\text{--}2 \times 10^6$ FMs of a single color were vortexed for 30 seconds, placed in an injection vial, then vortexed again immediately prior to and during injection. FMs were injected by flushing 10cc of heparinized saline over 30 sec through the injection vial into the left ventricle via the carotid artery catheter. A reference blood sample from the femoral artery was withdrawn into heparinized syringes at

0.97 ml/min using a calibrated withdrawal pump beginning ~10 sec before and continuing until 2 min after start of injection. Deionized water (10 ml) was added to the reference sample for hydrolysis of blood cells.

4.3. Tissue processing

At the end of each study, animals were sacrificed by pentobarbital overdose and perfusion-fixed with normal saline and 10% buffered formalin. Brains were removed and allowed to post-fix in formalin for at least 48 hours. Fixed brains were cut into 3 mm-thick coronal sections using our own custom-made piglet brain matrix. The matrix was formed from liquid plastic (Smooth-On, PA, USA) shaped around a normal average-weight 5-day-old piglet brain, with slats cut in the walls perpendicular to the neural axis and spaced 3mm apart center-to-center to guide the brain knife during cutting. Each section was embedded in 2 % agarose gel and cut by vibratome (Leica VT1000S, Leica Microsystems GmbH, Germany) into 100–200 μ m-thick slices, which were then mounted onto microscope slides using only gel mount (Biomedex, CA, USA) due to the thickness of sections.

Reference blood samples were filtered 3 times using 10 μ m-pore polycarbonate membranes (GE Osmonics, MN, USA) and once more using an 8 μ m-pore membrane to retrieve reference FMs. Filters were mounted on 75 $\times$ 50mm microscope slides (Corning, NY, USA) using gel mount and large 48 $\times$ 65mm coverglass (Gold Seal, Thermo Fisher Scientific, MA, USA).

4.4. Fluorescent imaging

All slides were imaged on an Alpha Array fluorescent slide reader (Alpha Innotech, CA, USA) at 15 μ m/pixel resolution using multiple excitation-emission filter pairs to isolate each FM color (Table 1). Each FM wavelength was captured as a separate gray-scale image of the entire tissue section (Figure 1a). Although the FMs themselves are 15 μ m in diameter, the radius of fluorescent illumination of a single FM into the tissue is 2–5 times greater. At 15 μ m/pixel resolution, FMs are detected as ~3–5 pixel-radius spots in tissue.

Narrow bandwidth optical filters and very short exposure times were required to minimize both auto-fluorescence of brain tissue and bleed-through of adjacent FM colors into a given channel. In our first two subjects, we used red FMs as one of the three colors. However, we found that this color was visualized in two of the channels – Cy3HF and Cy5HF. To correct the red bleed-through into the Cy3HF channel, the Cy5HF counts were subtracted from the Cy3HF counts to yield the final orange counts for these subjects. We replaced this color with crimson FMs for the last four subjects and had clear separation of all three colors (Figure 1c).

We determined the maximum tissue thickness through which the FM signal would penetrate for adequate imaging and counting of FMs by imaging tissue slices of different thicknesses with the slides face up and face down in each of the 3 channels. The counts face up and face down could then be compared, with loss of signal detected as unequal counts.

The blood filter slides were also imaged on the Alpha Array fluorescent slide reader at 15 μ m/pixel resolution. Since only a single FM color is present in the blood sample from a single timepoint, a single gray-scale image (Figure 1e) from only the matching excitation-emission filter pair (Table 1) was needed. The radius of fluorescent illumination from FMs on the filters was slightly smaller than in the tissue, detected as ~2–3 pixel-radius spots. This is due to differences in the optical properties of the filters compared with those of tissue.

4.5. Semi-automated image processing

FMs of each color were counted in tissue section and blood filter images by adapting a publically-available automated particle location program (see weblink in Crocker and Weeks; Crocker and Grier, 1996) written in IDL (ITT VIS, White Plains NY, USA). The program works by finding local maximums of intensity over a user supplied length scale. This value should be an odd integer slightly larger than the average particle size, but less than the average particle separation distance. We used a detection threshold value of 7 pixels for 15 $\mu\text{m}/\text{pixel}$ -resolution images. The program then identifies the number of potential particles within this range and plots brightness versus radius of the particles (Figure 1d). FMs can be easily identified as a band of high-intensity particles with radii between 2–5 pixels. The background tissue signal is usually an approximately Gaussian distribution of particles with intensities near zero and a peak around the threshold size. The number of particles identified as FMs were visually inspected (Figure 1b) and recorded for each image to a text file. Counting errors corrected by hand and were typically <5% of total counts.

To measure tissue area, a threshold intensity value is chosen just below tissue autofluorescent intensity in the FITC channel (closest channel to the maximum autofluorescent range of brain tissue = blue). To outline the total tissue area, contours are created on a binary image of pixel regions with intensity values above this threshold. High contours (values of 1 in the binary image) include both tissue and FM pixels. Low contours (values of zero in the binary image) are similarly generated for non-tissue regions (e.g. lateral ventricles) that are completely surrounded by tissue. A user-defined minimum size eliminates small non-tissue artifacts (e.g. dust) and prevents exclusion of tissue regions with minor fluctuations in intensity. Adding together the areas within all high contours and subtracting the areas within all low contours yields the total tissue area for a slice. This procedure is repeated to calculate the area of every tissue slice in a given section, and each area is multiplied by the thickness of the corresponding tissue to calculate volume of tissue (V). It is important to note that the intensity threshold will vary depending on elapsed time between tissue mounting and imaging, as older, drier tissue increases in auto-fluorescence intensity.

4.6. Data analysis

Absolute CBF ($\text{ml}/\text{min}/100\text{g}$) was calculated in each tissue region for each color as

$$CBF = \frac{ts \times rf \times 100}{rs \times V \times \rho}$$

where ts is the number of tissue microspheres, rs is the number of reference blood withdrawal microspheres, rf is the rate of the reference blood withdrawal (0.97 ml/min), V is the volume of the tissue region in cm^3 (calculated as described above) and ρ is the density of mixed gray-white matter brain tissue (1.04 g/cm^3) (Duck, 1990). The regions of interest were right and left cerebrum (each calculated from three representative 3mm coronal hemisections from frontal, parietotemporal, and occipital regions), as well as midbrain-pons, medulla, and cerebellum (each calculated from 3mm right and left coronal hemisections).

CBF measurements were reported as median (range) for each FM color in each brain region. A paired t-test was used to evaluate whether CBF differed in the cerebrum between right and left hemispheres. Nonparametric analyses using the Kruskal-Wallis test were used to compare CBF variation among brain regions and among microsphere colors. An additional Kruskal-Wallis test was used to compare orange regional CBF values between the animals that received crimson FMs and those that received red FMs.

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Abbreviations

CBF	cerebral blood flow
FM	fluorescent microsphere
MAP	mean arterial blood pressure

References

- Buckberg GD, Luck JC, Payne DB, Hoffman JI, Archie JP, Fixler DE. Some sources of error in measuring regional blood flow with radioactive microspheres. *J Appl Physiol* 1971;31:598–604. [PubMed: 5111009]
- Buckley NM. Maturation of circulatory system in three mammalian models of human development. *Comp Biochem Physiol A* 1986;83:1–7. [PubMed: 2868826]
- Crocker, JC.; Weeks, ER. <http://www.physics.emory.edu/~weeks/idl/>. Vol., ed.^eds
- Crocker JC, Grier DG. Methods of digital video microscopy for colloidal studies. *Journal of Colloid and Interface Science* 1996;179:298–310.
- De Visscher G, Haseldonckx M, Flameng W, Borgers M, Reneman RS, van Rossem K. Development of a novel fluorescent microsphere technique to combine serial cerebral blood flow measurements with histology in the rat. *J Neurosci Methods* 2003;122:149–56. [PubMed: 12573474]
- De Visscher G, Haseldonckx M, Flameng W. Fluorescent microsphere technique to measure cerebral blood flow in the rat. *Nat Protoc* 2006;1:2162–70. [PubMed: 17487208]
- Duck, FA. Physical properties of tissue : a comprehensive reference book. Academic; London: 1990.
- Glenny RW, Bernard S, Brinkley M. Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. *J Appl Physiol* 1993;74:2585–97. [PubMed: 8335595]
- Heymann MA, Payne BD, Hoffman JI, Rudolph AM. Blood flow measurements with radionuclide-labeled particles. *Prog Cardiovasc Dis* 1977;20:55–79. [PubMed: 877305]
- Leffler CW, Busija DW, Mirro R, Armstead WM, Beasley DG. Effects of ischemia on brain blood flow and oxygen consumption of newborn pigs. *Am J Physiol* 1989;257:H1917–26. [PubMed: 2513731]
- Luchtel DL, Boykin JC, Bernard SL, Glenny RW. Histological methods to determine blood flow distribution with fluorescent microspheres. *Biotech Histochem* 1998;73:291–309. [PubMed: 9888355]
- Moore LE, Kirsch JR, Helfaer MA, Tobin JR, McPherson RW, Traystman RJ. Nitric oxide and prostanoids contribute to isoflurane-induced cerebral hyperemia in pigs. *Anesthesiology* 1994;80:1328–37. [PubMed: 7516628]
- Powers KM, Schimmel C, Glenny RW, Bernards CM. Cerebral blood flow determinations using fluorescent microspheres: variations on the sedimentation method validated. *J Neurosci Methods* 1999;87:159–65. [PubMed: 11230812]
- Prinzen FW, Glenny RW. Developments in non-radioactive microsphere techniques for blood flow measurement. *Cardiovasc Res* 1994;28:1467–75. [PubMed: 8001033]
- Walter B, Bauer R, Gaser E, Zwiener U. Validation of the multiple colored microsphere technique for regional blood flow measurements in newborn piglets. *Basic Res Cardiol* 1997;92:191–200. [PubMed: 9226104]

Figure 1.

Fluorescent image processing example. Computer generated counts are overlaid in color without altering the original gray-scale captured images. **(a)** Gray-scale fluorescent image from a single channel (FITC) of a representative tissue section. **(b)** Image from (a) with FMs identified by the counting program corresponding to that channel ($\times$) overlaid with an offset up and to the right. **(c)** Image from (a) with counts from all 3 channels ($\times$ = yellow-green, $\times$ = orange, $\times$ = crimson) overlaid with offsets up and to the right of the FM in the tissue. Note that colors do not overlap, demonstrating good separation among channels. **(d)** Brightness-radius plot for image (a) including the 4-sided polygon delineating the high-intensity region corresponding to FMs. **(e)** Gray-scale fluorescent image from a single channel (FITC) of a representative blood filter. For images (a–c), brightness and contrast have been artificially enhanced for the purposes of visualizing the brain tissue in which the FMs are embedded.

Figure 2.

Box-and-whiskers plot of neonatal piglet cerebral blood flow (CBF, ml/min/100g) during normal resting conditions under isoflurane anesthesia by fluorescent microsphere color measured in the right cerebrum, left cerebrum, midbrain-pons, medulla, and cerebellum (median = dark line, 25th–75th percentile = box, range = whiskers).

Fluorescent microsphere colors used in this study, their excitation and emission wavelengths, and the corresponding fluorescent imager optical filter and exposure settings.

Table 1

FM Color	Excitation wavelength	Emission wavelength	Fluorescent channel	Excitation bandwidth	Emission bandwidth	Exposure time
Yellow-green	505 nm	515 nm	FTTC	455–495 nm	518–568 nm	8 ms
Orange	540 nm	560 nm	Cy3HF	527–552 nm	570–620 nm	8 ms
Crimson	625 nm	645 nm	Cy5HF	620–650 nm	665–705 nm	100 ms
Red	580 nm	605 nm	Cy3HF/Cy5HF	see above	see above	see above

Table 2

Physiologic conditions in neonatal piglets during each of the fluorescent microsphere injections.

	yellow-green	orange	red or crimson
HR (beats/min)	179 ± 27	176 ± 34	186 ± 30
MAP (mmHg)	58 ± 8	56 ± 10	52 ± 8
temperature (°C)	37.9 ± 0.6	38.2 ± 0.3	38.2 ± 0.4
pO₂ (mmHg)	124 ± 31	127 ± 29	137 ± 24
pCO₂ (mmHg)	52 ± 7	47 ± 7	48 ± 6

HR = heart rate, MAP = mean arterial pressure.

Table 3

Fluorescent microsphere counts of randomly selected 200 µm thick tissue slices fluorescently imaged face up (counts) and face down (flipped) in each of the 3 channels. None of the face up counts differed from corresponding face down counts by more than 10%, demonstrating good tissue penetration of fluorescent signal through 200 µm thick tissue.

Slide #	FITC counts	FITC flipped	Cy3HF counts	Cy3HF flipped	Cy5HF counts	Cy5HF flipped
1	10	10	6	6	8	8
2	16	16	15	16	5	5
3	9	9	11	12	12	12
4	20	20	12	13	21	21
5	11	11	30	31	19	19
6	15	14	19	21	6	6
7	148	150	71	70	19	20
8	155	155	64	64	22	21
9	171	166	75	77	23	23
10	89	89	27	25	2	2
11	103	105	28	27	1	1
12	71	70	30	29	0	0