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Quantitative fluorescence microscopy provides high resolution imaging of passive diffusion and P-gp mediated efflux at the *in vivo* blood–brain barrier

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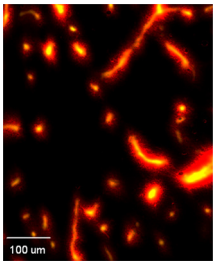
HIGHLIGHTS

- Quantitative fluorescence microscopy can determine the rate of dye movement across the blood–brain barrier.
- Rhodamine 123 is subject to efflux at the blood–brain barrier.
- Texas red appears not to be subject to efflux at the rat blood–brain barrier.
- Once past the blood–brain barrier, dye diffusion in parenchyma is not instantaneous.

GRAPHICAL ABSTRACT

Quantitative fluorescence microscopy provides significant insight into various fields. A novel method to calculate the blood to brain transfer rates of the dyes is presented. Rhodamine 123 is subject to p-glycoprotein mediated efflux and can be increased nearly 20-fold with p-glycoprotein inhibition. Once dyes have crossed the BBB, diffusion of the dye molecule is not instantaneous.



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ABSTRACT

Quantitative fluorescent microscopy is an emerging technology that has provided significant insight into cellular dye accumulation, organelle function, and tissue physiology. However, historically dyes have only been used to qualitatively or semi-quantitatively (fold change) determine changes in blood–brain barrier (BBB) integrity. Herein, we present a novel method to calculate the blood to brain transfer rates of the dyes rhodamine 123 and Texas red across the *in situ* BBB. We observed that rhodamine 123 is subject to p-glycoprotein mediated efflux at the rat BBB and can be increased nearly 20-fold with p-glycoprotein inhibition. However, Texas Red appears to not be subject to MRP2 mediated efflux at the rat BBB, agreeing with literature reports suggesting MRP2 may lack functionality at the normal rat BBB. Lastly, we present data demonstrating that once dyes have crossed the BBB, diffusion of the dye molecule is not as instantaneous as has been previously suggested. We propose that future work can now be completed to (1) match BBB transfer coefficients to interstitial diffusion constants and (2) use dyes with specific affinities to cellular organelles or that have specific properties (*e.g.*, subject to efflux transporters) to more fully understand BBB physiology.

1. Introduction

Historically, dyes have been integral in studying the blood–brain barrier (BBB). Notably, the restricted movement of dyes between the blood and brain compartments was first demonstrated by Paul Ehrlich in the early 19th century (Ehrlich, 1885). In this work, water-soluble dyes injected intravenously stained all organs except

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the brain and spinal cord. This was attributed to either the dye's lack of affinity for brain parenchyma or the presence of a physical barrier between the brain vasculature and brain tissue (Hawkins and Davis, 2005). Subsequent experiments by Goldmann demonstrated trypan blue injected into the cerebrospinal fluid (CSF) stained the brain and spinal cord but did not accumulate in the periphery, confirming the latter hypothesis (Goldmann, 1913). It is now known that trypan blue binds to albumin (Stopa et al., 2006), a 66 kDa plasma protein, and the resulting complex is too large to appreciably move across an intact BBB. Moving ahead 50 years, in 1967, Reese and Karnovsky demonstrated on an ultra-structural level that the BBB was a physical barrier for the blood to brain passage of horse-radish peroxidase (MW 40,000) (Reese and Karnovsky, 1967), because the spaces between adjacent brain endothelial cells were effectively sealed together preventing paracellular movement (Brightman et al., 1971).

At approximately the same time, the literature shifts from predominantly using dyes to document the presence of the intact BBB, to using dyes to qualitatively measure BBB disruption in various pathophysiological states. For example, in 1956, trypan blue was used to show that the BBB could be disrupted by ultrasonic damage (Bakay et al., 1956) which worsened in the presence of angiography imaging agents (Shealy and Crafts, 1965). Dye accumulation was also seen in circulatory arrest with prolonged resuscitation (Lin and Kormano, 1977), significant acute arterial hypertension (Johansson et al., 1970), seizures (da Costa, 1972; Nemeroff and Crisley, 1975), and radiation (Schettler and Shealy, 1970).

It was recognized very early vital dye studies were only qualitative. To overcome this, radiotracers such as ^{203}Hg were concurrently administered. This method provided an initial visualization of dye extravasation followed by quantitative measurement of BBB disruption (Dereymaeker et al., 1970; Schettler and Shealy, 1970; Shealy and Crafts, 1965). It should be noted that these initial studies simultaneously injected two different tracers to demonstrate size selective openings at the BBB (Shealy and Crafts, 1965). However, spatial resolution of dye distribution was lost. Quantification of BBB disruption using autoradiography quickly became the gold standard (Blasberg et al., 1983) and has evolved into well-designed double or triple labeled studies where size dependent BBB permeability changes can be simultaneously measured (Miyagawa et al., 2003; Uehara et al., 1997). Though autoradiography does have limitations, two or three tracers require weeks to months of film development followed by the subtraction of multiple signals to obtain data (Miyagawa et al., 2003). In addition, while spatial resolution of the tracer can be maintained, image resolution with traditional film autoradiography is typically limited to ~25–50 μm (Schmidt and Smith, 2005).

Recently, dye distribution across the BBB was completed using an *in situ* rat brain perfusion method (Hawkins and Egleton, 2006; Takasato et al., 1984). This work brought up an intriguing possibility that free dye movement across the BBB could be quantified in the absence of factors normally present in the blood which may alter apparent permeability coefficients. However, similar to previous reports using dyes, spatial resolution for quantification was not maintained even though microscopy was completed which allowed for qualitative visualization of permeability changes in different brain regions.

Given the recent developments in the field of quantitative microscopy (Dorn et al., 2008; Lockman et al., 2010), and that BBB disruption can be very heterogeneous depending on the insult (Baumbach and Heistad, 1985; Belayev et al., 1996; Brown et al., 2004; Nitsch and Klatzo, 1983), we set out to determine if we could expand previous studies of dye movement across the BBB using a quantitative approach, in which we couple *in situ* brain perfusions with quantitative fluorescence microscopy using post mortem analysis techniques similar to autoradiography. We hypothesize that using the brain perfusion technique, we could take advantage of differing dye properties [*e.g.*, dyes being a substrate for efflux transporters (Bachmeier and Miller, 2005; Fontaine et al., 1996; Wang et al., 1995)] and that we could resolve permeability variances in brain at ~1 μm increments.

2. Methods

2.1. Chemicals

Sulphorhodamine 101 (Texas Red: TxRd), Rhodamine 123 (R123) were purchased from Molecular Probes Invitrogen (Eugene, OR, USA). Probenecid, furosemide and verapamil were purchased from Sigma (St. Louis, MO). Cyclosporine A was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). MK571 was purchased from Cayman chemicals (Michigan, USA). All other chemicals used were of analytical grade and were used as supplied.

2.2. Animals

Male Fischer-344 rats (250–320 g) were purchased from Charles River Laboratories (Kingston, NY, USA) and were used for all the perfusion experiments done in this study. All studies were approved by the Animal Care and Use Committee and were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.3. Cell culture

Drug-sensitive MCF-7, adriamycin-resistant MCF-7/AdrR cells were kindly donated by Dr. US Rao, Texas Tech University Health Sciences Center, Amarillo, TX (Fairchild et al., 1990; Rao et al., 2006). MDCK-MPR2 cell line (Evers et al., 1998) was a kind gift from Dr. Borst (Netherlands). The cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin, 100 U/mL; and streptomycin, 100 μg/mL). Cells were grown in a 37 °C humidified incubator with 5% CO_2.

2.4. In vitro transport studies

For fluorescent microscopy experiments, cells were seeded in 24-well microplates at seeding density of 50,000 cells per plate. After 24 h, cells were washed in PBS and then incubated with R123 (1 μM) in the presence or absence of known P-gp inhibitors (verapamil, cyclosporin A) along with DAPI to stain the nuclei of cells. After a 30-min accumulation period cells were washed four times in PBS and then 0.5 mL PBS was added to each well. To prevent efflux of fluorescent dyes the plates were kept on ice until microscopic evaluations were performed. Cells were visualized with an inverted fluorescence microscope (Olympus IX81). Total intracellular R123 was determined using Slidebook 4.2 and normalized per 100 cells, counted with binary voxel masking using DAPI fluorescence as the reference.

Using nonlinear regression (Prism 5.0), the Hill equation was fit to the fold increase in intracellular fluorescence (relative to that observed in the absence of the inhibitor) as a function of increasing inhibitor concentration. The mean IC_{50} of each inhibitor was determined from at least 6 independent experiments. Unless otherwise stated, data are presented as mean ± S.E.M. Similar methodology was utilized for TxRd transport studies, where cells were incubated with TxRd (5 μM) in the presence or absence of MRP inhibitor MK571 along with DAPI.