Non-invasive measurement of retinal permeability in a diabetic rat model

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Funding information
The Medical Research Council, Grant/Award Number: MR/P003214/1 and MR/L01985X/1; Masonic Charitable Foundation; The National Eye Research Centre

Abstract
Objective: The gold standard for measuring blood-retinal barrier permeability is the Evans blue assay. However, this technique has limitations in vivo, including non-specific tissue binding and toxicity. This study describes a non-toxic, high-throughput, and cost-effective alternative technique that minimizes animal usage.

Methods: Sodium fluorescein fundus angiography was performed in non-diabetic and diabetic Brown Norway rats on days 0, 7, 14, 21, and 28. Sodium fluorescein intensity in the retinal interstitium and a main retinal vessel were measured over time. The intensity gradients were used to quantify retinal vascular permeability. Post-study eyes were fixed, dissected, and stained (isolectin B4) to measure required parameters for permeability quantification including total vessel length per retinal volume, radius, and thickness.

Results: In the non-diabetic cohort retinal permeability remained constant over the 28-day study period. However, in the diabetic cohort there was a significant and progressive increase in retinal permeability from days 14-28 (P < .01, P < .001, P < .0001).

Conclusions: This novel imaging methodology in combination with mathematical quantification allows retinal permeability to be non-invasively and accurately measured at multiple time points in the same animal. In addition, this technique is a non-toxic, rapid, sensitive, and cost-effective alternative to the Evans blue assay.

KEYWORDS
Fick’s Law, fundus fluorescein angiography, non-toxic, permeability, quantitative, retina, sensitive, vessel

Abbreviations: avi, audio video interleave; BBB, blood-brain barrier; BRB, blood-retinal barrier; DR, diabetic retinopathy; EB, Evans blue; EC, endothelial cell; FFA, fundus fluorescein angiography; GLUT2, glucose transporter 2; i.p., intraperitoneal; Na-Fl, sodium fluorescein salt; OCT, optical coherence tomography; PBS, phosphate buffered saline; PF, permeability to fluorescein; Pₛ, solute permeability to solute s; RPE, retinal pigmented epithelium; STZ, streptozotocin.

David O. Bates and Kenton P. Arkill contributed equally to the work.

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https://doi.org/10.1111/micc.12623
Selective permeable barriers are vital for organ function and homeostasis. Such barriers include the EC barrier of blood vessels, the BBB, the BRB, the blood-spinal cord barrier, and the blood-placental barrier. Selective transport of micromolecular substances across these barriers occurs under physiological conditions but they are relatively impermeable to macromolecules such as albumin. Evans blue (EB) dye has a high affinity for albumin and has a poor ability to cross selective permeable barriers under normal circumstances and remains predominantly within the blood circulation. The breakdown of the barrier, for example, via trauma or cytokine release, causes leak of dyes such as this across blood vessel walls including the BBB/BRB. When diseases such as diabetic retinopathy, sepsis, capillary leak syndrome, or cancer result in disruption of these barriers, there is an increase in vascular permeability and dyes such as EB may extravasate from the circulation into surrounding tissues. Accumulation of EB dye can be extracted from stained tissues and quantified by spectrophotometry. Evans blue is extensively used to estimate changes in vascular permeability in a number of models including in stroke, cerebral ischemia, skin (often referred to as a Miles assay), endothelial damage caused by trauma, and the breast cancer brain metastasis model. Evans blue has also been used to measure breakdown in the blood-spinal cord barrier and the BRB.

The EB assay is rapid and sensitive and has become a widely used method for estimating barrier integrity and vascular permeability. However, this methodology has a number of significant assumptions associated with its use which can lead to significant over-simplification of the research findings if unaccounted for. These include (a) the contribution of convective flux of albumin (and dye bound albumin) to the total flux varying from minimal to highly significantly dependent on hemodynamic parameters rather than barrier properties, (b) a substantive percent of free dye present in the animal following administration, resulting in flux being highly blood flow-dependent rather than barrier-dependent, (c) lack of specific binding to albumin, (d) injection of dye dissolved in physiological solutions affects the structure of the dye, (e) EB binds albumin when it is in the tissues, (f) change in tissue clearance can account for the changes, (g) problems with spectroscopic methods that have been used to estimate the amount of dye, and (h) in vivo toxicity. These issues have been highlighted many times and are extensively discussed elsewhere.

This study describes a non-toxic and high-throughput alternative technique to the EB assay in the retina. In combination with the Micron IV retinal imaging microscope system (Phoenix Research Systems), we have used FFA and a novel post imaging analysis methodology described below. This technique can be carried out in less than 15 minutes, is minimally invasive, and can be repeated in animals, resulting in a reduction of the number of animals required when compared to the EB assay, which takes 2-3 hours to carry out per animal and requires multiple cohorts of animals per time point. In addition, we have estimated that our novel methodology is significantly more efficient than the EB assay to perform.

2 | MATERIALS AND METHODS

2.1 | Animals ethics

Experimental animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and under a UK Home Office license (PPL 30/3184), at the University of Nottingham Biological Services Unit.

2.2 | Gender selection

Male Brown Norway rats, which are more susceptible to STZ-induced diabetes (250-300 g, Envigo), were included in this study.

2.3 | STZ dose and route of administration

A total of 40 male Brown Norway rats were weighed and given a single i.p. injection of STZ (Sigma-Aldrich). An additional 14 control rats were administered 300 μL saline i.p.

2.4 | Fast or non-fasted prior to STZ dosing

Fasting can be used to minimize competition between STZ and glucose for low-affinity GLUT2 transporters on β-cells. In the period following STZ dosing, the animals enter a hypoglycemic state and begin to show signs of sudden weight loss, polyuria, and dehydration. In our initial pilot study, animals were fasted overnight, prior to dosing in the morning, but their weights dropped dramatically during that period and this had a subsequent effect on weight loss in the proceeding study, exceeding a 20% weight loss moderate severity threshold. Therefore, in the subsequent study we did not fast prior to dosing. We ran the risk of the animals not becoming diabetic (~10% of cases) but this allowed the animals to feed, retain, or increase their weights prior to dosing and avoid the subsequent decline in health and weight otherwise observed. Animals with weights exceeding 300 g tolerated the 50 mg/kg STZ dose and maintained weights throughout the study or until the point of insulin supplementation.

2.5 | Sucrose enrichment

In addition to water, a 15% (w/v) sucrose solution was made available in a separate drinking bottle to alleviate the initial hypoglycemic spike following STZ induction. The volume of sucrose intake was monitored over a 72-hour period.
2.6 | Insulin supplementation

Current blood glucose control in diabetic rodent models focuses on maintaining the diabetic animal in a state of moderate hyperglycemia, with normal weight gain in the absence of severe ketonuria. This state can be achieved by once-daily injections of titrated insulin doses or by implantation of a continuous release insulin pellet. To reduce animal stress by repeated injections, we subcutaneously implanted one third of a single insulin pellet (LinShin). The 7-mm-long implant has a diameter of 2 mm and is designed to facilitate handling and insertion. Upon implantation, gradual erosion of the implant starts at once and the effect of released insulin on the blood glucose level can be detected in <1 hour. Unlike injectable insulin, the implant releases a set basal dose of insulin every hour. Therefore, an animal given an optimal implant dose should show no glucosuria and ketonuria, which are both difficult to prevent by daily insulin injections, due to the action of the insulin lasting only part of the 24-hour cycle.

2.7 | Blood glucose measurement

On days 0, 4, and prior to sacrifice (day 28), blood glucose levels were tested using a sample of blood taken from the tail vein and an Accu-Chek blood glucose monitor. Rats with blood glucose levels of >15 mmol/L and above were deemed hyperglycemic. Streptozotocin-injected rats that did not become hyperglycemic on day 4 were re-injected with STZ the following morning and subsequently evaluated for diabetes, as outlined above.

2.8 | Optimization of anesthetic knockdown

Due to the severity of the diabetic model and in combination with injectable anesthetics, gaseous anesthesia (halothane) was trialed. However, this had constraints. Firstly, the small animal gaseous mask sat just underneath the eye making it very difficult to align the retinal microscope with the eye without dislodging the gaseous mask and risking the animal becoming lucid. Secondly, gaseous anesthesia was not sufficient to prevent the eye from responding to the light source, blinking and rolling during imaging, without giving a high percentage of halothane. Injectable anesthetic removed the cumbersome equipment required for gaseous knockdown, the control of multiple flow meters, and ultimately stabilized the animal to prevent the eye from moving during FFA. We therefore used an injectable anesthetic regimen combining ketamine hydrochloride, 37.5 mg/kg (Zoetis) and medetomidine hydrochloride, 0.25 mg/kg (Produlab Pharma BV) i.p.

2.9 | Optimization of Na-Fl dose and volume

Na-Fl (MW 376.27) was prepared in sterile water, and 0.2 μm filtered, and 0.1, 10, 100 mg/mL dilutions were prepared and stored at room temperature and away from direct light until required. Intraperitoneal injections were administered in a non-injected lower quadrant of the abdomen while the anaesthetized rat was on the imaging cradle and the retina of the right eye had been previously located and aligned using the Micron IV. This was achieved by raising the right back leg, maintaining retinal alignment by minimal animal movement. The retinal imaging software was set to record prior to injection and continued to record until the dye had reached a saturation level in the retina (~3 minutes).

2.10 | Optimization of Na-Fl route of administration

Two routes of administration, intraperitoneal and intravenous, were tested with all three doses of Na-Fl. The tail was pre-warmed in a beaker of warm water (30-35°C) to dilate the blood vessels, and an intravenous injection was administered via the lateral tail vein. This method was more technically demanding and resulted in a sudden saturation of the retinal vasculature in approximately 30 s. In comparison, the i.p. route was simpler to administer and gave more gradual leakage of the dye into the retinal vasculature, over a period of approximately 3 minutes.

Animals were anaesthetized as previously described and transferred to an image cradle fitted with a heat mat. Viscoatears was applied to the cornea of both eyes to prevent dehydration, and whiskers were placed out of the field of view using a cotton tip. The Micron IV was advanced toward the cornea, and the optic nerve was centered in the field of view, altering the brightness and focus to achieve a crisp image of the main retinal vessels. Once the eye was correctly aligned, bright field fundus images of the retina were captured to check for any ocular abnormalities. The green filter was selected, and a 3-minute video footage of the retina was recorded at 15 frames per second. Animals then received a single 250 μL i.p. injection of Na-Fl. This was repeated on days 7, 14, 21, and 28 aligning the eye in the same position as captured on day 0 (Figure 1). Development of cataracts with resultant blurring of posterior segment view meant that some eyes (n = 2) were excluded from the consecutive FFA. Due to the rapid uptake and delayed elimination of Na-Fl from the animal’s circulatory system, only one eye could be imaged per session.
2.11 | FFA analysis

Angiograms were imported as avi files into Fiji software, and mean fluorescence intensity was measured in a major retinal vessel and nearby tissue (which includes unresolved capillaries) every 200 frames up to 2400 frames. An initial time course was plotted, and only the region where there was detectable tissue fluorescence but no major vessel saturation was used for analysis.

2.12 | Immunofluorescence

On day 28, animals were sacrificed and both eyes enucleated and immediately fixed in 4% PFA for 30 minutes. Retinae were excised from the scleral/choroidal cup and stained with an endothelial cell-specific marker, isolectin B₄ (lectin from Bandeiraea simplicifolia conjugated to biotin) followed by Alexa Fluor 488-streptavidin. Whole-mounted retinae were imaged using confocal microscopy (Leica TCS SPE) to generate z stacks, and allow for analyses of vascular structural parameters across the superficial and deep plexuses. Length per volume and mean radius were measured using Fiji software by manually drawing along all vessels using the freehand line tool. The distance between the upper and lower plexus was taken as the vascular retinal thickness.

2.13 | Statistical analysis

Unless otherwise stated, all data are shown as mean ± SEM. All data, and graphs were formulated with Microsoft Excel (Microsoft Office Software), GraphPad Prism v7/8 (GraphPad Software Inc), Fiji, and Imaris. Vascular spacing statistical analysis was calculated using a one-way ANOVA with Bonferroni’s correction, and 28-day permeability analysis was calculated using a two-way ANOVA with Bonferroni’s correction. All results were considered statistically significant at *P < .05, **P < .01, ***P < .001, ****P < .0001.

3 | RESULTS

To quantify the effective solute permeability ($P_s$) for the vascular wall, the parameter derivation in Appendix 1 was used, which is directly comparable to previous derivations from single capillaries. To enable this calculation to take place, the fluorescence intensity
was measured in one of the major retinal blood vessels and in a box outside the major retinal vessel containing no visibly distinct vessels (ie, no vessels larger than approximately 20μm in diameter, Figure 2A). The permeability to fluorescein ($P_{fluorescein}$) was estimated as described in Appendix 1 using FFA (Figure 2B). The calculations make the assumptions that the surface area available for exchange is the same in the diabetic animal as the control.

To determine these parameters tissue was stained to quantify the retinal vasculature. The retina has two clear plexuses (Figure 3A), one at the surface and one within the retinal layer. The vascular length (Figure 3B) and radius (Figure 3C) were calculated. There was no significant difference between the mean radius, the distance between the two plexuses (Figure 3D), nor the length of vessel per confocal volume.

We therefore calculated the permeability using cohort parameters and assuming that the surface area was unchanged throughout for each cohort. Figure 4A shows that while permeability remained constant in non-diabetic animals, there was a significant and progressive increase in permeability in diabetic animals.

To determine whether permeability could be linked, the data for all three diabetic and six controls over 28 days are shown (Figure 4B). These results show that the increase in permeability is progressive and that paired (linked) analysis can be used to determine a progressive increase in permeability with diabetic duration, allowing for a substantial reduction in number of animals per group as well as a reduction in overall numbers.

4 | DISCUSSION

These data show that the STZ model of diabetes (with insulin supplementation) in rats induces increased vascular permeability in the retina. This has been shown before via other methods and in other neuronal tissues, indicating a systemic response. However, of note is that the permeability increases early including a trend at day 7, reaching significance by day 14, that is, in early stages prior to gross vascular remodeling, as observed by measuring between the plexuses in the retina where there was no significant thickening (eg, by edema) of this layer. The FFA approach shown is practical, using repeated measures over a time course, that is, for therapeutic intervention, and could if desired be used in combination with other techniques such as OCT.

Leakage (increased solute flux from blood to tissue) can be caused by enhanced vascular permeability, increased driving force for flux (eg, enhanced concentration gradient), increased surface area, or hemodynamic changes driving convective flux of solute. In the eye, homeostasis of the retina is maintained by the low permeability to proteins and water of the BRB. The outer BRB comprised of tight junctions between the RPE cells separates the choroid from the neuronal retina, and recent evidence indicates that it is also broken down in diabetes and contributes to retinal and macular edema. Evidence presented here confirms that the inner BRB, formed of the retinal vascular wall, becomes more permeable in this rat model of diabetes, contributing to the development of DR.

The current gold standard model of permeability measurement in the eye is the EB assay developed by Xu et al. The EB assay can only be performed once per animal and therefore cannot be paired and, hence, to be able to determine changes in permeability that develop over a four-week period, would require eight animals per group at three separate time points each (ie, for three groups—control, diabetic, and diabetic + treatment) or a total of 72 animals. Eight animals per group are required because the measurements require the means of each group to be compared at each time point using a one-way analysis of variance, with post hoc analysis of each group resulting in 12 degrees of freedom ($n = 1$ animals × $n = 1$ groups = $6 \times 2$). In the methodology described here, the number per group can be reduced to a minimum of 3 because a two-way analysis of variance with a paired analysis

**FIGURE 3** Retinal vessel dimensions remain unchanged in non-diabetic versus diabetic Brown Norway rats. Average retinal vessel length per confocal volume, radius, and vascular retinal thickness were measured in non-diabetic (NDb) and diabetic (Db) cohorts prior to incorporating into the mathematical permeability equation. Eyes were enucleated at sacrifice, fixed, and hemisected and retinæ vessels stained (isolectin B4). (A). Total vessel length per confocal volume (B) and radius (C) measurements were calculated from non-diabetic and diabetic retina. (D) Vascular retinal thickness was measured from the upper to the lower plexus in the same animal cohorts. All data are shown as mean ± SE.
is used resulting in the same 12 degrees of freedom (3 time points, 
\( n = 1 \) groups, \( n = 1 \) animals). This results in a reduction in num-
bers of animals to \( 9 \) in total. Thus, the current methodology could 
reduce the animal numbers required to determine whether an in-
tervention reduces retinal permeability by eightfold which can be 
inferrable as an increase in methodological sensitivity.

The retina permits non-invasive and direct in vivo visualization 
of neuronal tissue and vasculature. Retinal imaging is an important 
diagnostic tool in ophthalmic disease, and changes in retinal vascula-
ure are used to determine the severity of systemic disease. Imaging 
can be undertaken with and without contrast agents. Na-Fl has been 
given intravenously since the mid-1900s to temporarily highlight 
retinal vessels and thus improving in vivo assessment of the retinal 
circulation and BRB integrity.\(^{25,26}\) Currently, Na-Fl is administered 
as a topical clinical tool for a host of conditions including diabetes,\(^{27}\)
glaucoma,\(^{26}\) vascular occlusions,\(^{28}\) neovascularization,\(^{29}\) and inflam-
matory disorders.\(^{30}\) With the advancement in wide-field fluorescein 
angiography, subtle changes in the peripheral BRB disruption can 
be detected.\(^{31}\) The principal of FFA is a series of collated images over 
time to demonstrate choroidal, arterial, and venous filling, and clear-
ance. Images are then visually examined to identify non-perfused 
areas or retained fluorescence that may indicate vascular compo-
mise as indicated by fluorescein leakage. However, intermittent 
timing and inaccurate timing limit the analysis of fluorescein images to 
a qualitative assessment. Vascular measurements that have been 
taken to date largely quantify the time it takes for NA-Fl to emerge 
in particular regions. While useful, this does not provide information 
on the extent of change in the barrier function of the vasculature in 
any given location.

More recently, Hipwell et al\(^{32}\) have designed a method to analyze 
an entire fluorescein angiogram. However, the analysis was limited 
to identifying the time to reach maximal fluorescence. Following on 
from this, Hui et al\(^{33}\) and colleagues have outlined a quantitative spa-
tial and temporal analysis of fluorescein angiography dynamics in a 
rodent model. Therefore, the sensitivity is also limited to detect sub-
tle changes such as differentiating vascular leakage from angiogen-
esis. However, video angiography could facilitate the quantitative 
analysis of more subtle differences in filling and leakage that occur in 
disease, for example, early DR.

## 5 PERMEABILITY QUANTIFICATION

Often permeability measurements determine solute flux, clear-
ance, or other parameters. When dealing with vasculopathies (eg, 
diabetic), this can be important as individual exchange vessels 
can become more permeable, but the wider tissue could receive 
less molecules, for example, due to fewer vessels. It is clear from 
Equation A17 that the FFA technique is sensitive to the difference 
in the tissue and vessel intensity components; however, this is non-
linear to the permeability so the ratio \( \frac{I_{p}}{I_{v}} \) or derivatives 
can only be used phenomologically to determine true permeability 
changes (ie, changes in the barrierc function of the vessel wall). In this 
STZ rodent model of diabetes, there were no significant differences 
for the vessel radius or exchange length but the result is sensitive to 
these parameters and our vascular assessment was not on the im-
aged volume but a similar area on the retina. We expect that if there 
was a wider understanding of the contextual vascular structure, pa-
ricularly between animals, or we could use the specific vessels used 
to measure \( P_{v} \) then it would tighten the point variation.

The \( P_{v} \) measured here is the effective \( P_{v} \) meaning that we have 
not taken into account capillary net pressure. The net pressure is 
the sum of the hydrostatic pressure out of, and the oncotic pressure 
into, the vessel. It is not currently possible to measure the pressure in 
the capillaries in the retina non-invasively, which can vary due to 
vascular shunting, local blood flow changes and global blood pres-
sure. To do so would require sophisticated optical detection and 
multiple fluorescent tracers: a large particulate tracer that can be 
imaged during flow, a large molecular weight molecule that cannot 
escape the vessels (or bright organic nanodots, for example\(^{34}\)), 
and a mid-sized molecule that can escape but not to the same extent as 
Na-Fl (353 Da in aqueous solution). Noting that this method is lim-
ited in the signal to noise in the measured tissue volume, brightness 
of the tracer will be an important practical factor without creating a
sharp bolus (ie, more invasive carotid injection). 10 kDa (eg, a chemokine) to 70 kDa (eg, albumin) molecules would be feasible sizes to test, if a stable version bright enough to observe orders of magnitude lower fluxes was achievable. Their flux fold change would be measurable yet likely markedly different to Na-Fl itself. The relative comparison may elude to which layers within the wall are disrupted in pathologies. This would provide a better understanding of the flow dynamics and size selectivity but would still not be able to determine vascular hydrostatic or osmotic pressures. The change of ratio between the mid-sized and Na-Fl molecules would allow a more detailed understanding of the specific vascular wall layer that is disrupted in DR pathology.

PERSPECTIVES

In summary, we highlight a simple, rapid, sensitive method for determining retinal permeability that can be used to drastically reduce the number of animals required to determine permeability changes in models of DR.

ACKNOWLEDGMENTS

This work was supported by The Medical Research Council MR/P003214/1 (KA); and MR/L01985X/1 (DOB, JW); The National Eye Research Centre (DOB); and the Masonic Charitable Foundation (DOB).

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APPENDIX 1

From Fick's Law:

\[ P_i = \frac{J_i}{S \cdot \Delta C} \]  
(A1)

where \( J_i \) is the flux of the number of molecules moving into the tissue \( \langle N_f \rangle \)

\[ J_i = \frac{dN_i}{dt} \]  
(A2)

\( S \) is the exchange area of the capillaries. \( \Delta C = \text{Difference in concentration between lumen } C_V \text{ and tissue } C_f \) but if we assume that initially \( C_f = 0 \) and throughout \( C_f \ll C_V \):

\[ \Delta C = C_V \]  
(A3)

If \( L_{tot} \) = total length and \( r = \text{mean radius of the vessels within the tissue imaging volume } V_j \) (see Figure 2A), the exchange area \( S \) within \( V_j \) becomes:

\[ S = 2\pi r L_{tot} \]  
(A4)

\( V_j \) has a total number of molecules \( (N_{V_j}) \) being the sum of the molecules in the tissue \( (N_f) \) and capillaries \( (N_c) \) within it:

\[ N_{V_j} = N_c + N_f \]  
(A5a)

\[ N_f = N_{V_j} - N_c \]  
(A5b)

Hence:

\[ \frac{dN_f}{dt} = \frac{dN_{V_j}}{dt} - \frac{dN_c}{dt} \]  
(A6)

When substituted in Equation A1 gives:

\[ P_i = \frac{\left( \frac{dN_{V_j}}{dt} - \frac{dN_c}{dt} \right)}{S \cdot C_V} \]  
(A7)

As all imaging volume \( V_z \) is encompassed by the major vessel, the mean number of molecules in the unit volume is \( C_V \).

\[ \frac{N_{V_j}}{V_z} = C_V \]  
(A8)

\[ \langle N_{V_j} \rangle = C_V \]  
(A9)

where \( <> \) denotes the mean over the respective volume. Further, the number of molecules can be assumed to be directly proportional to fluorescent intensity \( I \) measured throughout (ie, \( I \propto N \); \( I \) therefore substitutes for \( N \) to give:

\[ P_i = \frac{\left( \frac{dI_{V_j}}{dt} - \frac{dI_c}{dt} \right)}{2\pi r L_{tot} \cdot \langle I_{V_j} \rangle} \]  
(A10)

If we assume the capillaries have the same solute concentration as the major vessel then:

\[ I_c = x r^2 L_{tot} \langle I_{V_j} \rangle \]  
(A11)

Subsequently:

\[ \frac{dI_c}{dt} = x r^2 L_{tot} \frac{d}{dt} \langle I_{V_j} \rangle \]  
(A12)

Substituting Equation A12 into Equation A10 gives:

\[ P_i = \frac{\frac{dI_{V_j}}{dt}}{2\pi r L_{tot} \langle I_{V_j} \rangle} - \frac{r}{2} \frac{d}{dt} \langle I_{V_j} \rangle \]  
(A13)
The length of the vessels $L_{\text{con}}$ in the confocal volume $V_{\text{con}}$ (measured subsequent to euthanasia) contains all the vessel plexuses in the depth of field from the transport measurements, therefore:

$$L_{\text{tot}} = \frac{L_{\text{con}} V_1}{V_{\text{con}}} \quad \text{(A14)}$$

Substituting for $L_{\text{tot}}$ in Equation A13 gives:

$$P_s = \frac{V_{\text{con}}}{L_{\text{con}} V_1} \frac{1}{2\pi r} \frac{d \langle I_{V_1} \rangle}{dt} - \frac{r}{2 \langle I_{V_2} \rangle} \frac{d \langle I_{V_2} \rangle}{dt} \quad \text{(A15)}$$

But:

$$\frac{1}{V_1} \frac{d I_{V_1}}{dt} = \frac{d \langle I_{V_1} \rangle}{dt} \quad \text{(A16)}$$

So finally:

$$P_s = \frac{d \langle I_{V_1} \rangle}{dt} \frac{V_{\text{con}}}{2\pi r L_{\text{con}}} \left[ \frac{1}{\langle I_{V_1} \rangle} - \frac{r}{2 \langle I_{V_2} \rangle} \frac{d \langle I_{V_2} \rangle}{dt} \right] \quad \text{(A17)}$$

One of the difficulties with these experiments is finding the real $t = 0$. The tracer is via i.p. injection so takes time to reach the vasculature and travel via the heart and lungs to the retinal capillaries. Here $P_s^{t=0}$ is off the most interest as it fulfills the $\Delta C = C_t$ axiom. To extrapolate to $t = 0$ time was plotted against $<I_{V_2}>$ and $<I_{V_1}>$, $t = 0$ was defined as the intercept from the linear fits (Figure 2B). This was usually in the order of 20s different to frame timestamp. These linear fits were also used to calculate $P_s^{t=0}$ adjusting accordingly for the non-zero of the intensity intercept either from background or below the detection limit of the camera (this is not shown in Figure 2B). From $P_s^{t=0}$, an exponential fit (expected as $C_t$ increases) was applied to determine $P_s^{t=0}$. 