Am J Physiol Renal Physiol. 2014 May 1; 306(9): F1098–F1100.

PMCID: PMC5243215

doi: [10.1152/ajprenal.00652.2013](https://doi.org/10.1152/ajprenal.00652.2013)

Letter to the editor: "Quantifying albumin permeability with multiphoton microscopy: why the difference?"

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TO THE EDITOR: Utilizing intravital multiphoton microscopy, Schießl and Castrop (19) examined glomerular albumin permeability and the effect of angiotensin II. Recent data relating to glomerular albumin permeability and proximal tubule cell albumin handling have renewed the debate regarding the importance of both facets in albuminuria. The utilization of multiphoton microscopy to study this phenomenon has divided the community as initial studies by Russo et al. (13) reported a glomerular sieving coefficient for albumin (GSC_A) 50× greater than previously reported by techniques such as micropuncture (23). The model generated by these higher glomerular albumin permeability values relies on reabsorption and transcytosis of albumin and is gaining traction as numerous investigators have delineated proximal tubule (PT) proteins whose dysfunction results in albuminuria (5, 11, 18). Notably, three different investigators using diphtheria toxin receptor insertion into PT cells showed PT injury lead to a marked increase in albuminuria with normal glomerular light and electron microscopy morphology (5, 20, 25). In particular, Sekine et al. (20) (Fig. 3), Zhang et al. (25) (Fig. S4), and the review by Grgic et al. (5) (Fig. S4) show marked proteinuria and albuminuria, well into the nephrotic range, occurring temporally with PT dysfunction following one treatment with diphtheria toxin, and disappearing with PT recovery. There were no changes in glomerular morphology at either the light (25) or electron microscopic (5) levels.

Publications by Peti-Peterdi and Tanner in 2009 (10, 21), which were referenced by Scheißl and Castrop (19), sowed doubt and confusion about the GSC_A values reported by Russo et al. (12, 13). Briefly, Peti-Peterdi reported a low GSC_A value that was not measured using albumin but by a broadly dispersed 70-kDa rhodamine dextran (10). On closer inspection, this dextran generated a time-dependent GSC as the smaller molecular weight (MW) fractions were cleared out early, leaving larger MW fractions to produce increasingly lower GSCs (16). In the second study conducted by Russo et al. (12), molecular calibration using a narrowly dispersed 69.7-kDa dextran produced a GSC values of 0.025 ± 0.0041 , virtually identical to those obtained by Rippe's group using fractional clearance for the same sized dextran with similar dispersion characteristics (0.022) (1).

The current work of Schießl and Castrop (19) appears to have used settings causing this same lack of sensitivity produced when using higher offsets. Evaluation of a digital copy of Fig. 1B from their manuscript when it was downloaded, color decoded, blue channel selected, and thresholding was done of the corresponding blue region of the glomerulus revealed 87.3% of the pixels had a reading of zero. This would correspond to an offset of 42–45 in our study (17). This places the sensitivity well below the linear range for filtered albumin in an extrapolation to our study. (Note: the blue color warns the user the highlighted pixels have an intensity value of zero). To correctly adjust the offset, one should instead generate a “pre-” background image before infusion of a fluorescent compound and use those images to subtract the background values from identical image planes and regions taken postinfusion (15). The offsets should be set so that only a few pixels randomly display a value of zero (blue) and are not adjusted further.

Indeed, factors critical in measuring GSC_A were validated in our two previous studies (16, 17). Nuances in novel technologies such as the correct photodetector offset, critical in multiphoton microscopy, can lead to generation of erroneous data when inappropriately set. These parameters are not widely understood and must be identified and better established in future studies.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.M.S. and B.A.M. drafted the manuscript; R.M.S. and B.A.M. prepared figures; R.M.S. and B.A.M. edited the manuscript; R.M.S. and B.A.M. revised the manuscript; R.M.S. and B.A.M. approved the final version of the manuscript.

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Figures and Tables

Fig. 1.

Photodetector offset influences glomerular albumin permeability values dramatically. The offset or “black level” settings determines the lower detection limits on the photomultiplier tube (PMT) detectors found in many laser scanning confocal and multi-photon microscopes. *A*: effect on detector sensitivity using 2 different offsets on a range of background subtracted Texas red albumin (TRA) solutions *in vitro*. The average intensity readings from the images were divided by the concentration of TRA to give a direct unit of sensitivity. This unit should be constant across the concentration range if sensitivity is unaffected, as shown when offset is set to 38. Using a higher numerical offset of 45, which forces background values closer to zero, markedly decreases the microscope's ability to correctly detect fluorescence in identical dishes. With detector sensitivity decreased particularly at lower TRA concentrations, the sensitivity value is no longer constant across the range for offset 45. *B*: list of background subtracted, glomerular sieving coefficients for albumin (GSC_A) collected at different offsets for the same set of glomeruli. Note the rapidly decreasing GSC_A seen starting at *offset* 42. There is a 262-fold decrease in GSC_A in a comparison of *offsets* 38 and 52. *C* and *D*: identical images of a glomerulus after infusion of TRA, taken at *offsets* 38 and 45, respectively. The blue color seen in Bowman's space in *D* (*offset* 45) indicates the values located therein have a value of zero before background subtraction. Colors alerting the user to pixels in the image having intensity values above or below the detection limit of the detectors are standard in every microscope system (typically red for saturated values and blue or green for values at zero). The GSC for TRA in these experiments, where only the offset was changed for the image, averaged 0.015 $\pm$ 0.0047 using *offset* 38 and 0.00114 $\pm$ 0.00096 using *offset* 45. Values in this, and all of our studies, were background subtracted, with a reference image taken at the corresponding offsets.

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