



## RESEARCH ARTICLE

# REVISED A new analysis approach for single nephron GFR in intravital microscopy of mice [version 3; peer review: 2 approved, 2 not approved]

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## Abstract

**Background:** Intravital microscopy is an emerging technique in life science with applications in kidney research. Longitudinal observation of (patho-)physiological processes in living mice is possible in the smallest functional unit of the kidney, a single nephron (sn). In particular, effects on glomerular filtration rate (GFR) - a key parameter of renal function - can be assessed.

**Methods:** After intravenous injection of a freely filtered, non-resorbable, fluorescent dye in C57BL/6 mice, a time series was captured by multiphoton microscopy. Filtration was observed from the glomerular capillaries to the proximal tubule (PT) and the tubular signal intensity shift was analyzed to calculate the snGFR.

**Results:** Previously described methods for snGFR analysis relied on two manually defined measurement points in the PT and the tubular volume was merely estimated in 2D images. We present an extended image processing workflow by adding continuous measurement of intensity along the PT in every frame of the time series using ImageJ. Automatic modelling of actual PT volume in a 3D dataset replaced 2D volume estimation. Subsequent data analysis in R, with a calculation of intensity shifts in every frame and normalization against tubular volume, allowed exact assessment of snGFR by linear regression. Repeated analysis of image data obtained in healthy mice showed a striking increase of reproducibility by reduction of user interaction.

**Conclusions:** These improvements in image processing and data analysis maximize the reliability of a sophisticated intravital microscopy technique for the precise assessment of snGFR, a highly relevant predictor of kidney function.

## Open Peer Review

Reviewer Status

	Invited Reviewers			
	1	2	3	4
<b>version 3</b> (revision) 10 Jun 2021				 report
<b>version 2</b> (revision) 12 Apr 2021		 report	 report	 report
<b>version 1</b> 26 Nov 2020	 report	 report		

- Bruce Molitoris**, Indiana University School of Medicine, Indianapolis, USA
- Christopher Schmied** , FMP Berlin, Berlin, Germany
- Georgina Gyarmati**, University of Southern California, Los Angeles, Los Angeles, USA
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**Keywords**

Intravital Microscopy, 2-Photon Microscopy, Kidney, Single Nephron GFR, ImageJ, R

Any reports and responses or comments on the article can be found at the end of the article.



This article is included in the **NEUBIAS - the Bioimage Analysts Network gateway**.

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**Author roles:** **Kessel F:** Conceptualization, Formal Analysis, Methodology, Software, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Kröger H:** Data Curation, Investigation, Validation, Writing – Review & Editing; **Gerlach M:** Conceptualization, Resources, Supervision, Writing – Review & Editing; **Sradnick J:** Supervision, Writing – Review & Editing; **Gembardt F:** Writing – Review & Editing; **Todorov V:** Supervision, Writing – Review & Editing; **Hugo C:** Funding Acquisition, Project Administration, Resources, Supervision, Writing – Review & Editing

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**REVISED Amendments from Version 2**

Figure 1 was insufficiently described in the figure caption and hard to understand without the context of the entire article. In the new version we added more information to the figure caption to address this issue.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Glomerular filtration rate (GFR) is a key parameter of kidney function and deviations from normal GFR are a hallmark of renal diseases<sup>1,2</sup>. GFR describes the filtration of substances from blood in the glomerular capillaries, to the primary urine in the tubular system of the kidney. Therefore, changes in GFR serve to monitor disease progression<sup>1,2</sup>. GFR is also measured in animal models to study effects of pharmacological intervention on kidney function<sup>3</sup>. Advances in intravital imaging and multiphoton microscopy allow repetitive assessment of GFR and morphological changes in the smallest functional unit of the kidney – the nephron<sup>3-5</sup>. Longitudinal imaging of single nephrons (sn) enable direct correlation of structural and functional data<sup>3-5</sup>.

After intravenous injection of the freely filtered, non-resorbable, fluorescent dye LuciferYellow (LY), a time series was captured by multiphoton microscopy. Filtration was observed from the glomerular capillaries to the proximal tubule (PT) and the tubular signal intensity shift is analyzed to calculate the filtration rate. Translated to an image processing task, this can be generalized as the flow rate in a tube. Previous methods for this analysis<sup>3,4</sup> relied on two manually annotated measurement points in the PT and stereotypic estimation of PT volume in 2D images. Since results we obtained with this approach were highly variable, we expanded the analysis of image data via 3D modelling with open source software, to increase overall reproducibility and reliability of the analysis when comparing renal function of different experimental groups.

## Methods

### Animal experiments

Animal experiments were performed in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) Guidelines for the Care and Use of Laboratory

Animals and the Federal Law on the Use of Experimental Animals in Germany and approved by the ethical review committee at the Landesdirektion Sachsen (license DD-24.1-5131/338/37). For microscopy, male, 10–12 week old C57BL/6 mice were prepared as previously described<sup>5,6</sup>. In brief, a titanium abdominal imaging window (AIW) covered with a coverslip is surgically implanted above the kidney. The kidney is glued to the coverslip with cyanoacrylate glue before securing the AIW by tightening the skin in the AIW groove. Microscopy was performed one day after AIW implantation.

A custom-built temporary intravenous catheter (polyethylene tubing #587360 by Science Products GmbH with 0.3×12mm needle) was placed in the lateral tail vein. Fluorescent dyes were administered into the tail vein prior (Hoechst, AngioSpark) or during (LuciferYellow) microscopy (detailed information in [Table 1](#)).

All efforts were made to ameliorate harm to animals. Imaging (including injections of the fluorescent dyes) and the implantation is done under isoflurane anaesthesia. The image data of the five animals presented for the comparison of the extended workflow with the previous workflow in this manuscript were generated previously as part of an independent experiment (license DD-24.1-5131/338/37).

### Microscopy

Imaging was performed on an upright Leica SP8 multiphoton laser scanning microscope at the Core Facility Cellular Imaging. Settings for signal acquisition are summarized in [Table 2](#).

### Image and data analysis

Image processing and analysis was done in ImageJ<sup>7-9</sup> (1.53c) with 3D ImageJ Suite<sup>10</sup> and Bio-Formats<sup>11</sup> for the use of 3D image processing plugins and the Bio-Formats Importer. Data analysis was performed in R<sup>12</sup> (4.0.2), with RStudio<sup>13</sup> (1.2.5033) including ggplot2<sup>14</sup> (including dependencies) installed as additional library. The script executed the ImageJ macro from command line and subsequently analyzed and visualized the results. A detailed description of the algorithm is associated with the scripts on GitHub<sup>15</sup>.

The line region of interest (ROI) set for the extended workflow to manually define direction and position of the proximal

**Table 1. Dyes.**

Dye	Order Number	Supplier	Purpose	Application details	Channel	Excitation	Acquisition
AngioSPARK 680	NEV10149	PerkinElmer	Vessel dye	30 µl	3	860 nm	685–695 nm
Hoechst 33342	H3570	Thermo Fisher	Nuclear dye	50 µl (2 mg/ml)	4	860 nm	415–474nm
Lucifer Yellow CH dilithium salt	L0259-25MG	Sigma Aldrich	Freely filtered fluorescent dye	20 µl via syringe pump in 1 s (5 mg/ml)	2	860 nm	500–550nm

tubule (PT) was also used to determine the two measuring points (beginning and end of line) for analysis of image material based on the previously described approach<sup>3,4</sup>. Tubular diameter was calculated as the mean of five manually measured diameters.

**Results**

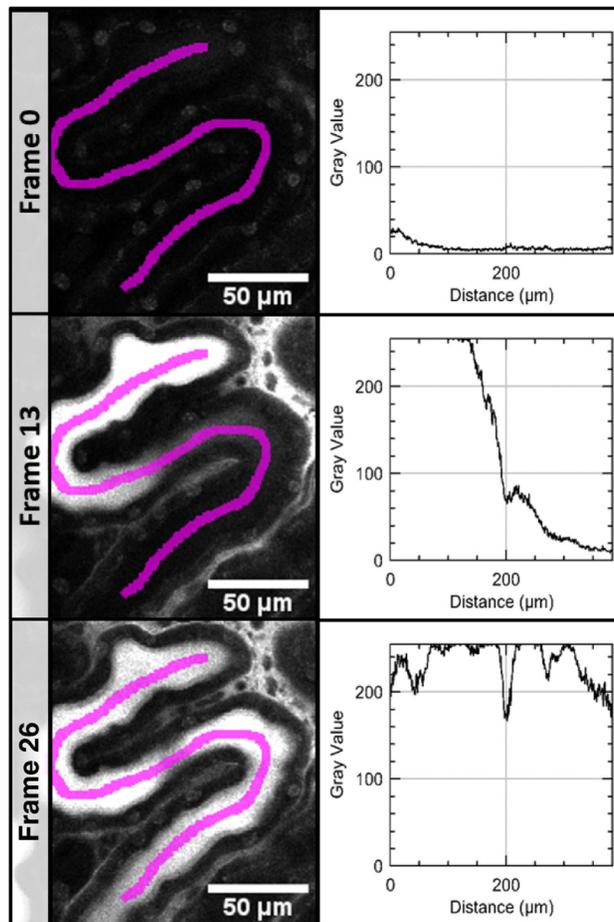
In the time series acquired after application of LY, a line ROI was set to manually define the position and direction of the

measurement. Along this ROI, x-y plots measured the dye intensity in the PT in every frame (Figure 1) and numerical results were saved.

For the automatic 3D modelling of PT volume the z-stack of the same field of view was acquired. Additional channels (Ch3: AngioSpark - vessels, Ch4: Hoechst - nuclei, Figure 2A) were subtracted plane by plane from Ch2 (target channel,

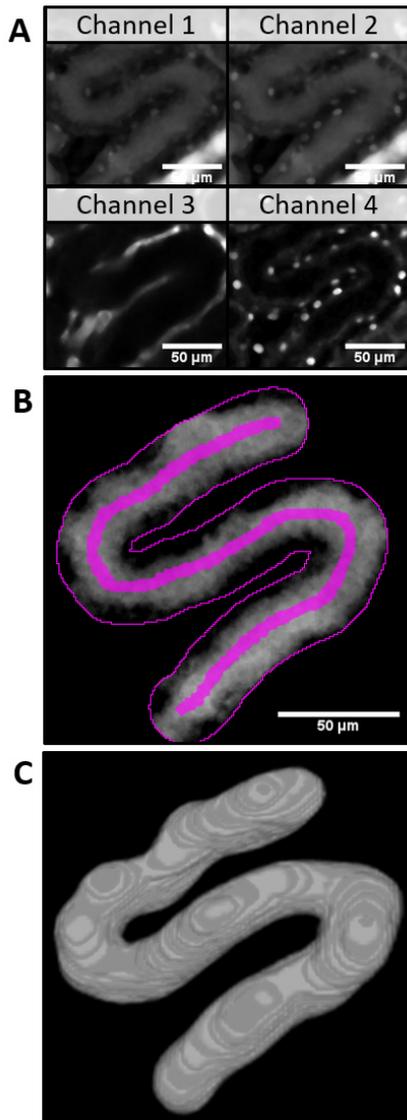
**Table 2. Image acquisition settings.**

Dye	Excitation	Objective	Resolution	Detection
AngioSPARK 680	860 nm, Chameleon II (Coherent)	40x 1.1 NA water immersion objective	Pixel size: 0.8513 µm frame rate (time series): 6 fps Voxel depth (z-stack): 1 µm	685-695 nm, HyD detector (Leica)
Hoechst 33342				415-474nm, PMT detector (Leica)
Lucifer Yellow CH dilithium salt				500-550nm, HyD detector (Leica)

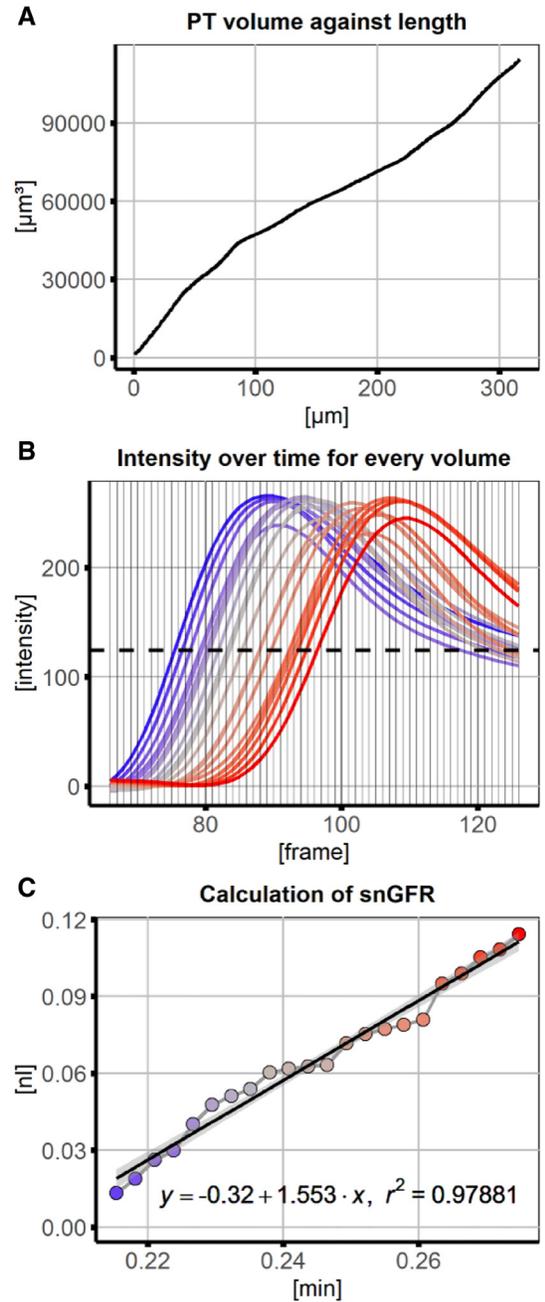


**Figure 1. Measurement of signal intensity in a time series of the proximale tubule (PT).** Signal intensity of LuciferYellow (LY) was measured along a line region of interest (ROI, magenta) in every frame (here only frame 0 - before LY injection, frame 13 and 26 are shown on the left). For visualization of the resulting data, the signal intensity along the ROI is plotted for these sample frames on the right. As the LY moves through the PT, the measured signal intensity shifts as well.

LY intensity) to remove spectral bleed-through artifacts (Figure 2B). With the 3D watershed, the PT was segmented (Figure 2C, 3D-model) and saved for visual verification. The cumulative PT volume was measured over the distance along the ROI and plotted in subsequent data analysis (Figure 3A). The position is now recalculated to the cumulative PT volume at each point along the ROI. From intensity measurements a threshold intensity was set to the turning point of fluorescence intensity over time at every volume (maximum slope, Figure 3B). The volume with this intensity was approximated in each frame and used for linear regression (Figure 3C, intersect of



**Figure 2. Automatic 3D modelling of tubular volume in a z-stack of the proximal tubule (PT).** **A**) After applying a 3D median filter, the channel 3 and channel 4 z-stacks were subtracted from channel 2 to eliminate spectral bleedthrough artifacts (**B**). The proximal tubule (PT) was segmented with the help of a 3D watershed (3D model of the resulting z-stack, **C**).



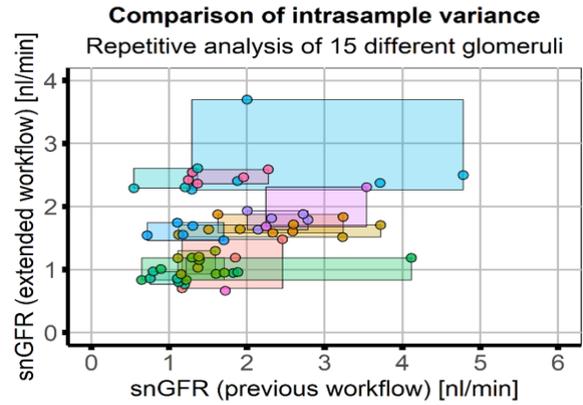
**Figure 3. Data analysis and linear regression of signal volume against time for calculation of glomerular filtration rate (GFR).** **A**) For every position along the line region of interest (ROI), the cumulative volume was measured, providing a conversion of position to volume. **B**) Numerical data underlying the x-y plots was saved and used to subsequently plot changes of signal intensity over time for every position (and converted to cumulative volume) along the line ROI. The dashed line represents the threshold value at which the corresponding volume of the proximal tubule (PT) was approximated for every frame. **C**) Using linear regression the snGFR could be calculated as the volume with the intensity threshold at the frames of interest (after conversion from μm³ per frame to nl per minute). Regression line is displayed with 95% confidence interval. The colour codes for the position along the PT (blue – beginning, red – end).

horizontal threshold at every frame with intensity curves). The slope of the regression line equals the snGFR after conversion of  $\mu\text{m}^3$  per frame to nl per minute. Together with information about PT length, PT volume and R-squared the results were summarized and saved in a data table.

Repeated analysis (five times) of 15 individual glomeruli by the same researcher showed that results obtained with the presented workflow had higher consistency (lower intrasample variance, CV=10.35%) compared to the previous approach (CV=38.75%, Figure 4). Due to the high variance with the previous approach a direct correlation of the workflows was not possible; however, the final result - the mean snGFR - was comparable (previous workflow:  $1.71\pm 0.91$ , extended workflow:  $1.70\pm 0.78$ ) and a two-sample Kolmogorov-Smirnov test of both result vectors showed that the distributions were not statistically different ( $p=0.4662$ ). Numerical results of the repeated analysis with both workflows are listed in Table 3.

### Conclusions

The progressive development of microscopy techniques like measurement of snGFR in experimental animals needs to be accompanied by improvements in analysis algorithms to use their full potential. In this manuscript we present a workflow by extending an existing analysis method via 3D modelling, for increased reproducibility, accuracy, but also transparency in the measurement of snGFR. By reducing user interaction, intrasample variance was markedly improved.



**Figure 4. Application and comparison of the workflows in image data of healthy mice.** Image data of healthy mice (five animals, 15 glomeruli) was analysed five times by the same researcher using the previous and the extended workflow. Scatter plot of results of the previous (x-axis) and extended workflow (y-axis) with rectangles used to indicate the range of results obtained in one glomerulus. Colours indicate data obtained from individual glomeruli. Intrasample variance with the extended workflow (variance along the y-axis, mean CV=10.35%) was smaller than with the previous workflow (variance along the x-axis, mean CV=38.75%). Both analysis workflows showed similar results (mean snGFR, previous workflow:  $1.71\pm 0.91$ , extended workflow:  $1.70\pm 0.78$ ) and a two-sample Kolmogorov-Smirnov test of both result vectors showed that the distributions were not statistically different ( $p=0.4662$ ).

**Table 3. Numerical data of repeated analysis with the previous and the extended workflow.**

	Previous Workflow			Extended Workflow		
	Mean	Standard deviation (SD)	Relative SD [%]	Mean	SD	Relative SD [%]
Dataset 1	1.783	1.057	59.254	1.724	0.132	7.675
Dataset 2	2.476	0.581	23.451	1.611	0.075	4.676
Dataset 3	2.296	1.123	48.900	2.587	0.125	4.842
Dataset 4	0.606	0.211	34.862	1.128	0.147	13.012
Dataset 5	1.441	0.207	14.355	1.012	0.151	14.968
Dataset 6	1.871	1.367	73.039	0.987	0.127	12.873
Dataset 7	0.995	0.204	20.535	0.851	0.077	9.078
Dataset 8	1.039	0.433	41.661	2.600	0.304	11.708
Dataset 9	2.732	1.456	53.306	2.648	0.593	22.375
Dataset 10	1.200	0.356	29.644	1.600	0.116	7.254
Dataset 11	2.393	0.347	14.505	1.811	0.113	6.265
Dataset 12	1.359	0.666	48.997	2.987	0.124	4.139
Dataset 13	1.628	0.460	28.242	2.477	0.091	3.665
Dataset 14	3.746	1.603	42.805	0.717	0.176	24.566
Dataset 15	0.176	0.084	47.642	0.814	0.067	8.225

Additionally, the automatically saved user input and intermediate results (z-stack of watershed of PT as shown in [Figure 2C](#) and graphs in [Figure 4](#)) for every analyzed dataset provide full possibility to verify every analysis step. These results can be used to objectively evaluate the measurement. Although the snGFR in this manuscript was very low for healthy animals compared to previously published values<sup>3</sup>, the range was comparable in both methods and not an artifact produced by the workflow but more likely caused by the general experimental setup.

Taken together, this workflow extension contributes to an overall improvement of the interpretation of snGFR measurements. Applied to experimental data this can cumulate in a higher power to detect statistically significant differences between experimental groups and even decrease the necessary sample size, thus having an impact on animal welfare.

## Data availability

### Underlying data

Zenodo: Sample dataset - cont-3D-snGFR. <https://doi.org/10.5281/zenodo.4275596><sup>16</sup>.

This project contains the following underlying data:

- Sample\_Dataset\_cont-3D-snGFR.tif (Sample file with time series and z-stack of three different glomeruli after injection of LuciferYellow for the analysis of single nephron GFR)
- Results.zip (Sample file for the selection (ROI sets) of the proximal tubulus in the sample dataset, including the resulting measurements (text files) in the time series and 3D modelling of the proximal tubules (tiff files))

- Graphs\_2020-09-30.zip (Intermediate results and graphs (png files) as obtained from the sample dataset with selections and measurement data in the results file)
- 2020-09-30-Result\_summary.txt (Final summary (text file) of calculated single nephron GFR for the three sample glomeruli based on selections from the results file)
- Dataset1.tif (Image data used for the comparison of previous and extended workflow in [Figure 4](#), includes 15 time series and the corresponding z-stacks)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

## Software availability

Source code available from: <https://github.com/NephrologieDresden/cont-3D-snGFR>

Archived source code at time of publication: <https://doi.org/10.5281/zenodo.4642427><sup>15</sup>.

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## Acknowledgments

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<http://www.doi.org/10.5281/zenodo.4275596>

# Open Peer Review

Current Peer Review Status:    

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## Version 3

Reviewer Report 16 June 2021

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### Anna Schueth

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I have no further comments to make.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Multiphoton microscopy and Light-sheet microscopy with application to mouse modes.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 2

Reviewer Report 07 June 2021

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### Anna Schueth

Department of Cognitive Neuroscience, Faculty of Psychology & Neuroscience, Maastricht University, Maastricht, The Netherlands

Here the authors are presenting an intravital multiphoton microscopy study to study the single

nephron (sn) and glomerular filtration rate (GFR) in mice. In the results section the authors describe an extended image processing workflow with ImageJ/Fiji and "R". Conclusively, this study shows an improved manner for image processing in order to study and analyse snGFR. The study seems technically sound to me.

However, Figure 1 is to my opinion not optimal. If separated from the main manuscript text, it is difficult to understand the presented results in this figure. Therefore, I would advise the authors to revise the figure description.

**Is the work clearly and accurately presented and does it cite the current literature?**

No

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

I cannot comment. A qualified statistician is required.

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Multiphoton microscopy and Light-sheet microscopy with application to mouse models.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 08 Jun 2021

**Friederike Kessel**, University Hospital Carl Gustav Carus at the Technische Universität Dresden, Fetscherstraße 74, Dresden, Germany

We thank Dr. Schüth for their reviewer report and the helpful remarks on Figure 1 and the insufficient figure captions. We agree that without the context of the entire article, the caption should contain additional information.

We address this issue in a new version of the manuscript and hope Figure 1 is now more understandable.

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 18 May 2021

<https://doi.org/10.5256/f1000research.55759.r84810>

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### Georgina Gyarmati

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Since multiphoton microscopy and other imaging approaches have become more commonly used technologies, the need for standardized and reproducible image analysis methods are much needed. Therefore, the authors' work is timely and important. The authors in this paper addressed the intra-observer variability of single nephron glomerular filtration rate (SNGFR) measurement and provided an extended workflow for image processing and data analysis to maximize reproducibility. The application of the extended workflow resulted in reduced variability of measurements, however, no significant difference was found between results obtained with the conventional and the new method.

The article is based on a false claim and hypothesis that the current published methods for SNGFR measurement is unreliable and produce high variability. Therefore, its scientific validity is questionable. There seems to be one major and one minor issue with this work. The major issue concerns the unclear overall significance of this technical advance because it provides only a minor incremental advance in the field. The claim that the conventional workflow does not provide reproducible and reliable results is not valid. There are many advantages of the conventional manual analysis method and when performed correctly by the published methods and meticulous observer the results are highly reproducible and have low variability. It is puzzling how the application of the conventional and new approach can produce over 5-fold differences in results from the same sample (Table 3). In my opinion, there are more stressing issues with the measurement of SNGFR, that would warrant the development of such advanced analytical tools. For example, SNGFR is not constant over time as compared to global GFR due to vasomotor (myogenic) tone and tubuloglomerular feedback (TGF). This results in significant alterations in GFR on the single nephron level by every 5-10 seconds (myogenic) or 30-50 seconds (TGF). Therefore, this is a more important issue in SNGFR variability than intra-observer variability in the analysis of a single timepoint SNGFR measurement.

The minor issue is the quality of the preparations shown in the supplement material as previously raised by Dr. Molitoris. There seems to be a significant amount of blebs and cell debris flowing in many tubular segments. All numerical SNGFR values shown in table 3 are below the physiological range. These suggest that the animals were not in physiological healthy conditions. Using these

datasets for the current analytic development work is not optimal.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Renal pathophysiology, intravital imaging

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Reviewer Report 23 April 2021

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**Christopher Schmied** 

FMP Berlin, Berlin, Germany

The authors addressed my raised concerns and significantly improved the documentation by supplying a good manual for the workflow. This manual now addresses the important prerequisites of the workflow, how to install any dependencies and how to use the workflow effectively. This should enable users to easily pick up the analysis and sort out any usage problems that might arise. Particularly the description of the results and intermediate files now contain very good comments to interpret and troubleshoot the analysis. Well done!

Thank you for also clarifying some parts of the manuscript and figures as well as providing further citations.

**Is the work clearly and accurately presented and does it cite the current literature?**

No

**Is the study design appropriate and is the work technically sound?**

No

**Are sufficient details of methods and analysis provided to allow replication by others?**

No

**If applicable, is the statistical analysis and its interpretation appropriate?**

No

**Are all the source data underlying the results available to ensure full reproducibility?**

No

**Are the conclusions drawn adequately supported by the results?**

No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Bioimage analysis, Computer vision, Data science

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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Version 1

Reviewer Report 28 January 2021

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**Christopher Schmier** 

FMP Berlin, Berlin, Germany

The authors describe a semi-automated analysis for measuring single nephron glomerular filtration rate (snGFR). An important parameter for assessing renal function. Intravital microscopy was used to record the filtration of a fluorescent dye along glomerular vessels to the proximal tubule. The aim of the analysis is to measure the flow rate. To achieve this the user first sets a line

ROI to determine the position and direction for the measurement of the intensity change across the time course. Then the entire 3D volume is segmented in a separate z-stack. The analysis is smart and tries to use all available information in their experimental setup to ensure a robust analysis. The generation of the 3D volume based on a 3D watershed uses the information from different channels to ensure a robust segmentation. Using a regression analysis makes sure that not only 2 points alone will contribute to the final measurement of the snGFR. I think this is a good image and data analysis approach to reduce measurement variability and increase statistical power.

The workflow runs as R script that also calls a Fiji macro. The interaction runs via sequential GUI prompts. This increases the ease of use.

The article is, in general, well written and contains most of the important information to understand the method and how it compares to previously used methods. The detailed description of the algorithm is sometimes a bit confusing but one can understand the rational of the authors. My main problem is with the lack of documentation that allows one to access and implement the tools. Here are my points for revision:

**MAJOR:** The algorithm makes sense. The description in the text and figure legends is however a bit hard to understand.

This sentence is particularly unclear: "*The position with this intensity was approximated in each frame and used for linear regression (Figure 3C)*". I guess what the authors wanted to express is that the volume was approximated at this position. Then the approximated volume was plotted over time and based on that linear regression was performed?

### **Figure 3 B&C and their legends are rather confusing:**

Figure 3B mixes in the volume of the segmentation, although this is not the message of the figure (Intensity and computation of threshold on slope). The color code of Figure 3B&C corresponds I guess to the positions? This is not explained anywhere. In Figure 3A first  $\mu\text{m}^3$  is used and then in Figure 3C nl?

**MAJOR:** I downloaded the material and it took me about 4 tries to get the scripts to work correctly. Here are the key impediments:

1. Does not execute on ubuntu 20.04: the R script uses functions that only work under Windows. This limitation needs to be explicitly stated. This also abolishes the advantage of cross platform tools such as Fiji and R.
2. The 3D watershed is not explicitly stated as dependency of Fiji. It needs to be clearly stated what needs to be installed and how.
3. The direction of the ROI is important but this was not clear from the documentation.

**MAJOR:** The Documentation word file provided is not helpful for actually using the scripts. It rather contains a code documentation that has some directions of using the program included. People with little expertise have no clear guidance for the usage and the important settings of the usage are entirely lost in all the detail.

1. The usage needs to be documented separately from the code.

2. The actual interaction with the program needs to be documented also via screenshots.

3. The important settings need to be explained clearly and in sufficient detail.

**MAJOR:** That one needs to draw the ROI in the direction of the wave was not really obviously documented or it got lost in the complexity of the code documentation. Please use screenshots or describe with words.

**MAJOR:** How the data needs to be acquired and structured for this workflow to function is not explained anywhere. The prompt for selecting a corresponding z-stack made initially zero sense. Since it was not clear that the .lif file must contain the multichannel time series AND the z-stack. Are the channels settings hard coded then? If the analysis is inflexible in its data input (which can be ok), it needs to be mention explicitly as an important prerequisite.

**MAJOR:** Reproducing the workflow using the provided .lif file resulted mostly in snGFR that were in a similar range. But still off. Maybe drawing the ROIs seems to be still an important source of variability. It would be good if there would be an easy way to load and visualize the ROIs provided by the authors. This shows easily how the authors intend users to set ROIs. One can load them via the ROI Manager during the GUI interactions but this produces an error later on:

Composite selections cannot be converted to lines. in line 520:

```
(called from line 193)
run ( "Area to Line" <)>
```

Maybe it would also be good to document in words along example screenshots how one best should set the ROI.

**MAJOR:** I am not in the kidney field. Maybe certain statements are common knowledge there and it is practice not to cite them. But the following statements in the introduction would strike me as requiring citations:

- *"Therefore, changes in GFR serve to monitor disease progression."*
- *"GFR is also measured in animal models to study effects of pharmacological intervention on kidney function."*
- *"Advances in intravital imaging and multiphoton microscopy allow repetitive assessment of GFR and morphological changes in the smallest functional unit of the kidney – the nephron."*
- *"Longitudinal imaging of single nephrons (sn) enable direct correlation of structural and functional data."*
- *"Since results we obtained with this approach were highly variable."*

**MINOR:** All the result files produced by the workflow and how to interpret them and recognize issues are not described anywhere in the documentation.

**MINOR:** It would be nice, at least in the documentation, to layout graphically the flow of the

workflow.

**MINOR:** Please include a brief description of the usage in the README. What needs to be installed (also the 3D watershed update site) and how to run the workflow. Also any important prerequisites should be mentioned there as well.

**MINOR:** Selecting the "executable Fiji file" needs to be described better and documented with a screenshot. Users that have Fiji preinstalled or rarely use Fiji will not know this.

**MINOR:** Figure 4 is hard to interpret and one cannot easily compare own results for reproducing the workflow. The result of the automatic analysis are included as an extra file. It would be nice to have the results of this automatic and manual analysis available as a table for a direct comparison.

**MINOR:** I miss an explicit point of contact or means of support for any users such as github, forum or Email.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

No

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Bioimage analysis, Computer vision, Data science

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 28 Mar 2021

**Friederike Kessel**, University Hospital Carl Gustav Carus at the Technische Universität

Dresden, Fetscherstraße 74, Dresden, Germany

First of all we thank the reviewer for the extensive report and constructive feedback. We agree that a lack of documentation, guidance and also support information notably impaired the usability of the workflow. In this context, the patience of the reviewer to implement the algorithm is highly appreciated.

Since most of the remarks were directly linked with lack of documentation, we uploaded detailed instructions to the GitHub repository and updated the associated release on Zenodo. This documentation now includes:

- A paragraph on the structure of the raw data
- System requirements (operating system)
- Software requirements (ImageJ, including update sites, R and RStudio with additional libraries)
- Instructions on how to run the workflow with screenshots
  - Explicitly pointing out the importance of the direction of the line ROI
  - With additional screenshots of example line ROIs for the images included in the sample dataset
- Information on all output files (ROI sets, result files, graphs)
- Suggestions for data interpretation and troubleshooting
- Contact information

Since this analysis was only recently developed and experiences when applying it to different image data are still limited we are determined to continuously expand the documentation and troubleshooting suggestions. We recognize that there is also room for improvement for the programming itself, regarding the limitation to Windows and hard-coded requirements of the raw data. We plan to support the gradual expansion of the workflow to be more adaptable – and applicable – in the future (as mentioned in the documentation).

Since the reviewer pointed out that some of the descriptions in the manuscript and figure legends were hard to understand, we rephrased some points. We hope it is now more understandable.

- Description of Figure 3C: Approximation of the volume for every position and plotting against time for linear regression
- Legends for Figures B and C: Colour code
- Conversion of units:  $\mu\text{m}^3$  to nl in Figure 3C

We also included the table with the numerical results as shown in Figure 4.

Finally, the statements on GFR and methods in intravital microscopy in the introduction can be supported with references that were already used in other contexts in the manuscript. Therefore, we additionally refer to them in the introduction.

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 16 December 2020

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### **Bruce Molitoris**

Department of Medicine, Indiana University School of Medicine, Indianapolis, USA

Measurement of SNGFR is an important undertaking and adding a 3D component to tubular volume is interesting. However, viewing glomeruli in mice of 10-12 weeks is not possible without significant invasive procedures. Ureteral obstruction has been used by some, but this author uses removal of 1 mm of cortical tissue to get down to cortical glomeruli. They do not say this but reference an existing paper. Since you can only see up to 100 microns with the 2-photon scope with high resolution, it is difficult to imaging tissue injury is not altering function of the glomerulus and tubules. This has to be discussed and if controls are available they should be mentioned. In the referenced paper the sieving Coefficient for albumin was very high and likely due to tissue injury. No glomerulus is shown in the present work and yet the authors indicate they followed from glom and then along the tubule. Were they measuring flow in S1 or S2 segments as flow would vary due to reabsorption?

Also, the subtraction of 3D volumes from each other for background subtraction is not recommended. It is best to subtract each individual plane from the corresponding channels.

It would also be helpful if they put figure 4 data into a table for easier and direct comparison.

#### **Is the work clearly and accurately presented and does it cite the current literature?**

Partly

#### **Is the study design appropriate and is the work technically sound?**

Partly

#### **Are sufficient details of methods and analysis provided to allow replication by others?**

No

#### **If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

#### **Are all the source data underlying the results available to ensure full reproducibility?**

No source data required

#### **Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Nephrology, imaging

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 25 Jan 2021

**Friederike Kessel**, University Hospital Carl Gustav Carus at the Technische Universität Dresden, Fetscherstraße 74, Dresden, Germany

We thank Prof. Molitoris for his time and the detailed review. However there seems to be a misunderstanding of the intentions of the submitted manuscript.

We present and validate a workflow and an algorithm to process image data obtained from single nephron GFR measurements by intravital microscopy. The measurement itself is not the focus of the manuscript. A detailed protocol on animal preparation and image data acquisition was merely cited (1), however the experimental setup is reproducible using these protocols. In our setup, superficial glomeruli can be imaged in the intact kidney of 10-12 week old animals with 2-photon microscopy with sufficient quality. We want to specifically point out that removing parts of the kidney cortex prior imaging is not included in the cited protocols and we did not perform this in our study either. Prof. Molitoris is possibly referring to the paper of Kidokoro et al. (2) which was cited by us in the context of illustrating an existing analysis approach for image data obtained from the snGFR measurement.

With the focus of the manuscript in mind: We compare results obtained from the analysis of the same image datasets with two different workflows (but using the same segments of the proximal tubule) – and not different experimental groups of differently treated animals. With all raw image data (openly accessible at Zenodo), the source code (openly accessible at GitHub) and open source software (ImageJ and R) the data we present are completely reproducible.

We agree with Prof. Molitoris that presenting more image data in the manuscript might be beneficial. The challenges of depicting time series and complex 3D image data in a 2D representation led us to the decision to upload all raw image data (time series and 3D datasets) to Zenodo, where it can be freely accessed. Lastly we rephrase the description of one of the image processing steps: As Prof. Molitoris pointed out correctly, the subtraction of one z-stack from the other is performed plane by plane, and not with 3D volumes.

Clarifying changes in the manuscript on the emphasis of the intention (presenting an image analysis workflow) will be made in an upcoming version. We would gladly invite Prof. Molitoris to review the updated manuscript again with this perspective.

1. Kidokoro K, Cherney DZI, Bozovic A, *et al.*: Evaluation of Glomerular Hemodynamic Function by Empagliflozin in Diabetic Mice Using In Vivo Imaging. *Circulation*. 2019;

**140**(4): 303–315.

2. Schiessl IM, Fremter K, Burford JL, *et al.*: Long-Term Cell Fate Tracking of Individual Renal Cells Using Serial Intravital Microscopy. *Methods Mol Biol.* 2020; **2150**: 25–44.

**Competing Interests:** No competing interests were disclosed.

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