# Introduction

Intravital microscopy in animal models is an emerging technique in life sciences with advanced applications in kidney research. Observations about morphological and functional changes of the smallest functional unit in the kidney – the nephron – can be done longitudinally throughout the progression of disease models in live mice. In particular, it is possible to assess structural features of a nephron and directly correlate it to its glomerular fitration rate (GFR), a key parameter of kidney function.

After intravenous injection of a freely filtered, non-resorbable, fluorescent dye a time series is acquired by multiphoton laser scanning microsopy. Filtration is observed from the intraglomerular capillaries to the connected proximal tubulus and filtration is calculated after analysis of the intratubular signal intensity shift.. Translated to a simple image processing task, this could be generalized as the assessment of the flow rate in a tube. Previous methods for this analysis relied on two manually set measurement points in the tubulus and the overall tubular volume was merely estimated based on length and diameter in 2D images [1, 2]. However, the results we obtained with this approach were highly variable, especially in tubuli with high curvature.

We extended the workflow in ImageJ [3, 4] by adding continuous measurement along the entire proximal tubulus in x-y-plots of fluorescence intensity in every frame of the time series. Additionally, automatic modelling of actual tubular volume in a 3D dataset replaced the volume estimation, hence increasing robustness, accuracy and objectivity . Subsequent data analysis in R [5, 6, 7] included smoothing of x-y intensity plots, calculation of intensity shifts in every frame and normalization against tubular volume for exact assessment of single nephron GFR (snGFR) by linear regression.

[1] Kang, J. J. et al. (2006). "Quantitative imaging of basic functions in renal (patho)physiology." Am J Physiol Renal Physiol 291(2): F495-502.

[2] Kidokoro, K. et al. (2019). "Evaluation of Glomerular Hemodynamic Function by Empagliflozin in Diabetic Mice Using In Vivo Imaging." Circulation 140(4): 303-315.

[3] Schindelin, J. et al. (2012). "Fiji: an open-source platform for biological-image analysis." Nat Methods 9(7): 676-682.

[4] Schindelin, J. et al. (2015). "The ImageJ ecosystem: An open platform for biomedical image analysis." Mol Reprod Dev 82(7-8): 518-529. [5] RStudio: Integrated Development Environment for R. R. Inc. Boston, MA, http://www.rstudio.com/.

6] R Foundation for Statistical Computing (2017). R: A Language and Environment for Statistical Computing. R Core Team. Vienna, Austria, https://www.R-project.org/.

[7] Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York.

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The workflow was developed and tested by Friederike Kessel with the help of Hannah Kröger in the lab of Prof. Christian Hugo Experimental Nephrology, Department of Internal Medicine of the University Hospital Carl Gustav Carus Technical University Dresden. It is published in the NEUBIAS gateway of F1000Research:

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## The raw data

In this workflow, microscopy data obtained with a Leica SP8 is analyzed. Raw data is stored in .lif-files that can contain a series of multichannel images, but must always contain both: a time series and the corresponding z-stack of every glomerulus. The time series are identified by the term „LY“ in the name, the z-stack by the term „PT“. Check the sample dataset on Zenodo (Friederike Kessel, Hannah Kröger, & Christian Hugo. (2020, September 17). Sample dataset - cont-3D-snGFR (Version 1.0.1). Zenodo. http://doi.org/10.5281/zenodo.4275596) for clarification. Important: The z-stack has to be acquired in the same field of view and with the same resolution (x-y) as the time series. Additionally, the settings for the channels are currently hard coded (Channel 2: Lucifer Yellow, Channel 3: Vessel Dye, Channel 4: Nuclear Dye). We would be happy to help you adapt the workflow for application on differently structured raw data, feel free to contact us!

## Requirements

Due to system commands specific to Windows Operating Systems, the scripts in this repository only work with Windows Operating System (tested with Windows 7 and 10). We would be happy to expand this and provide different versions to be tested with different operating systems upon request. Please contact us!

Programs necessary for the execution of the scripts are:

**Fiji – a distribution of the image processing software ImageJ** (https://imagej.net/Fiji/Downloads)

To ensure access to the most recent versions of additionally needed plugins in ImageJ, following update sites have to be added:

- 3D ImageJ Suite
- Java-8
- ImageScience
- Bio-Formats

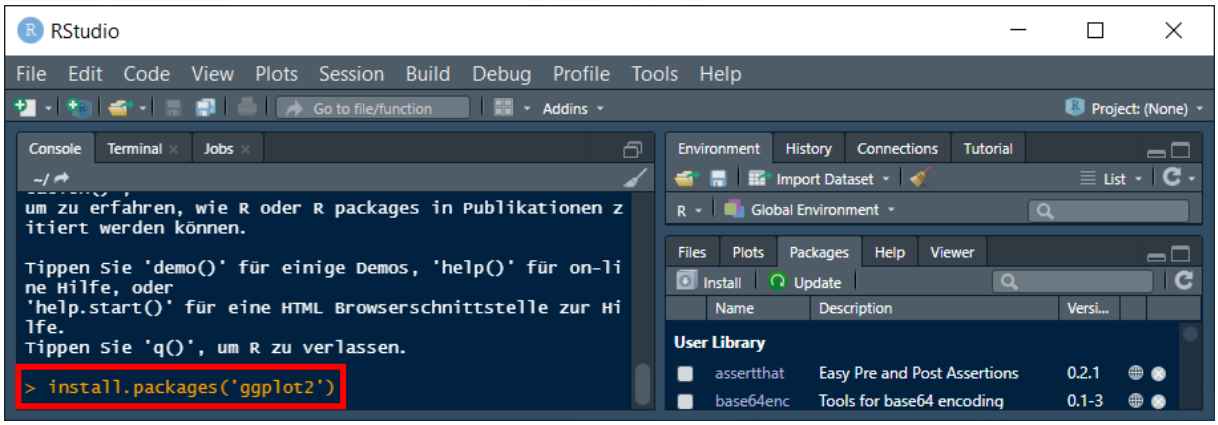
If you don’t know how to add update sites:
https://imagej.net/How_to_follow_a_3rd_party_update_site

**R – for statistical computation and data analysis** (https://cran.r-project.org/mirrors.html)

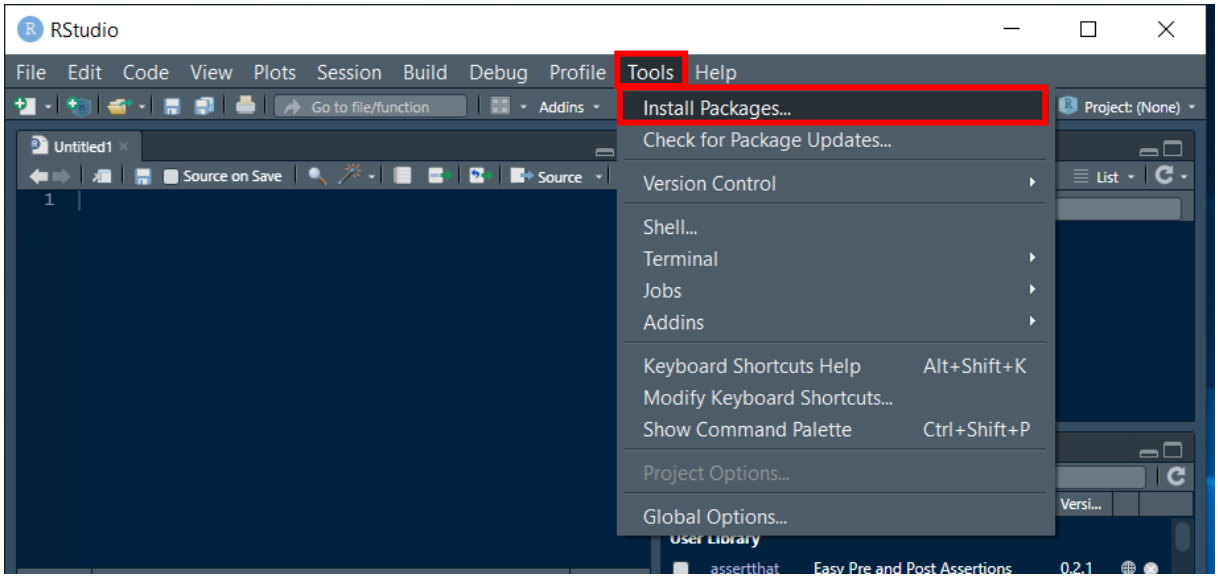
**RStudio – an additional development environment for R**
(https://rstudio.com/products/rstudio/download/)

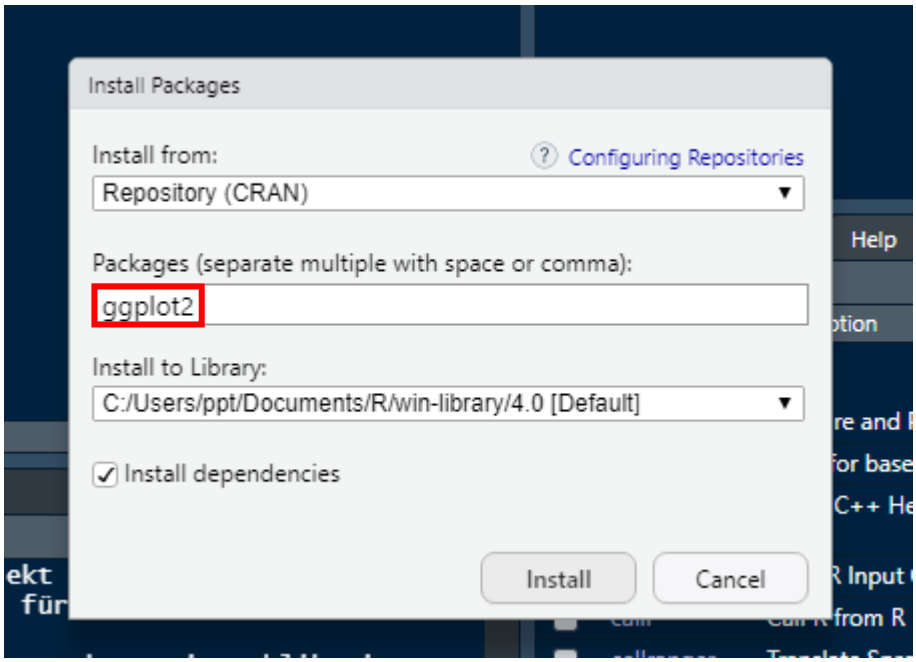
For the visualization of results in R, the package „ggplot2“ is required. To install this, execute the command

install.packages(„ggplot2“)



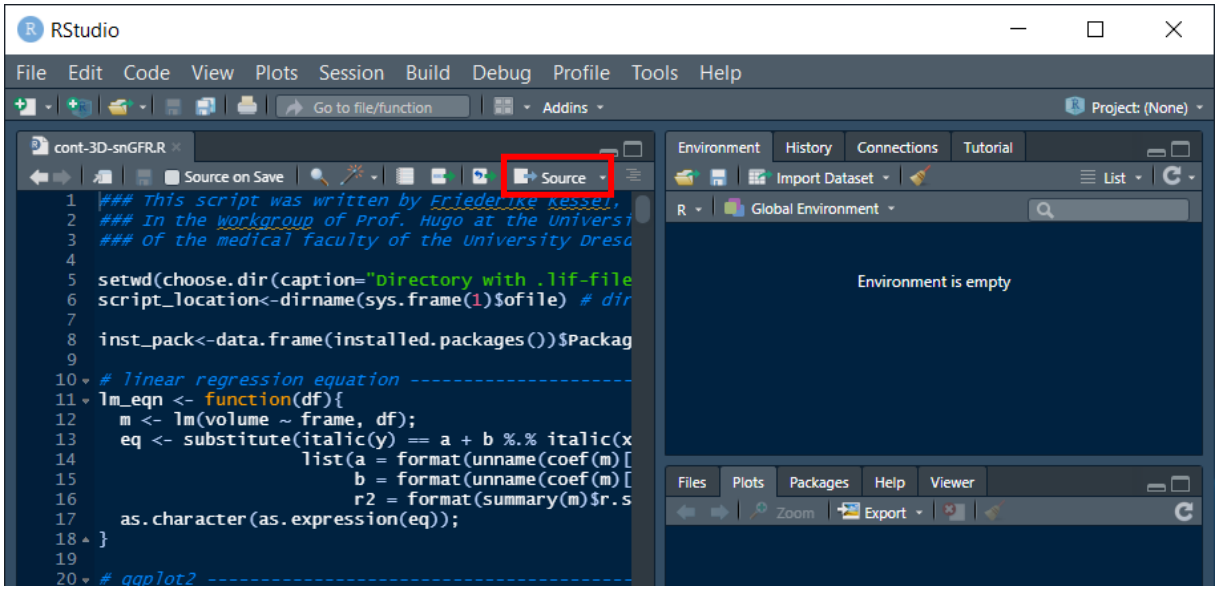

or install it by using the install packages GUI in RStudio.



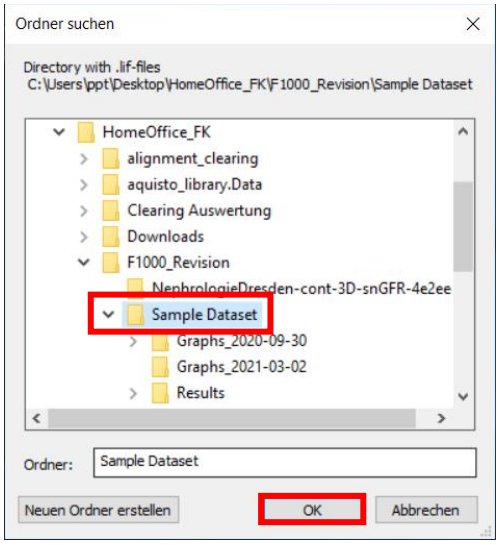



# Applying the workflow

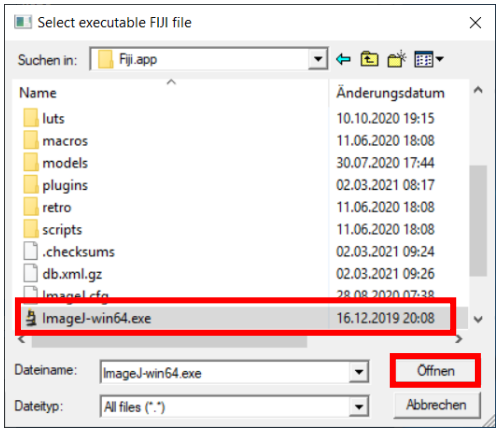
- Open R script (cont-3D-snGFR.R) in RStudio
- Run from source



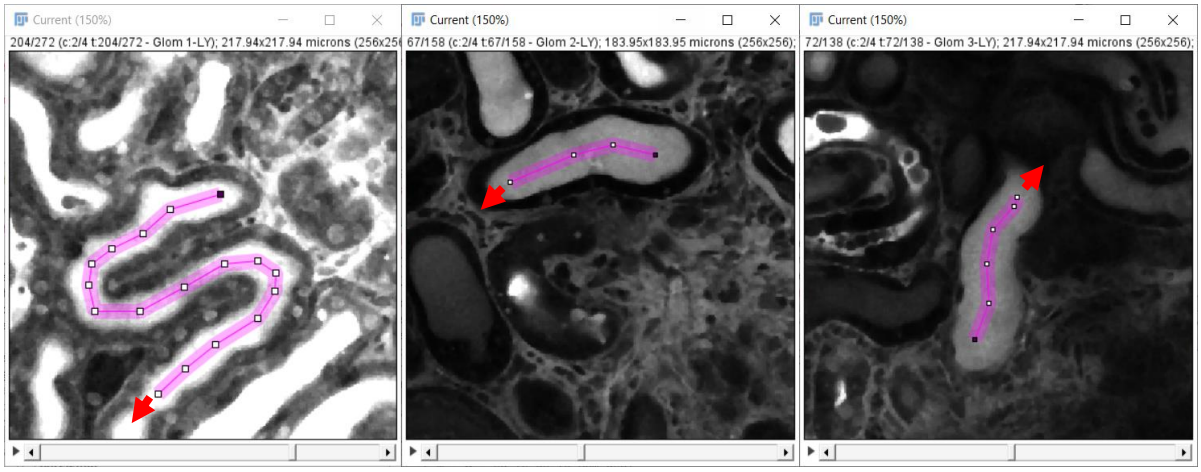

- Select directory with all .lif files that should be processed



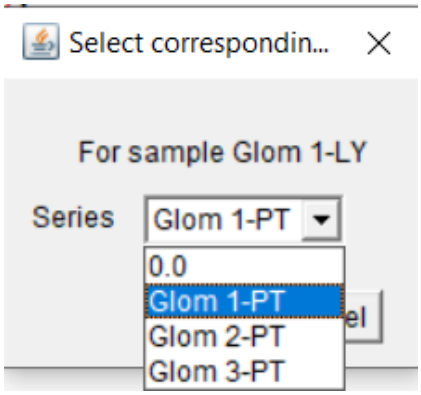

- Select executable Fiji file



- ImageJ is executed and the macro is called
- The Macro loops through all .lif files within the previously selected folder
    - In every .lif file the macro loops through every time series (indicated by LY in name)
    - Manual selection of line ROI is necessary (no composite selections, just one line in direction of flow – from the glomerulus to the end of the PT)
    - Examples for setting the line ROI in the sample dataset (red arrow indicates direction of line ROI)

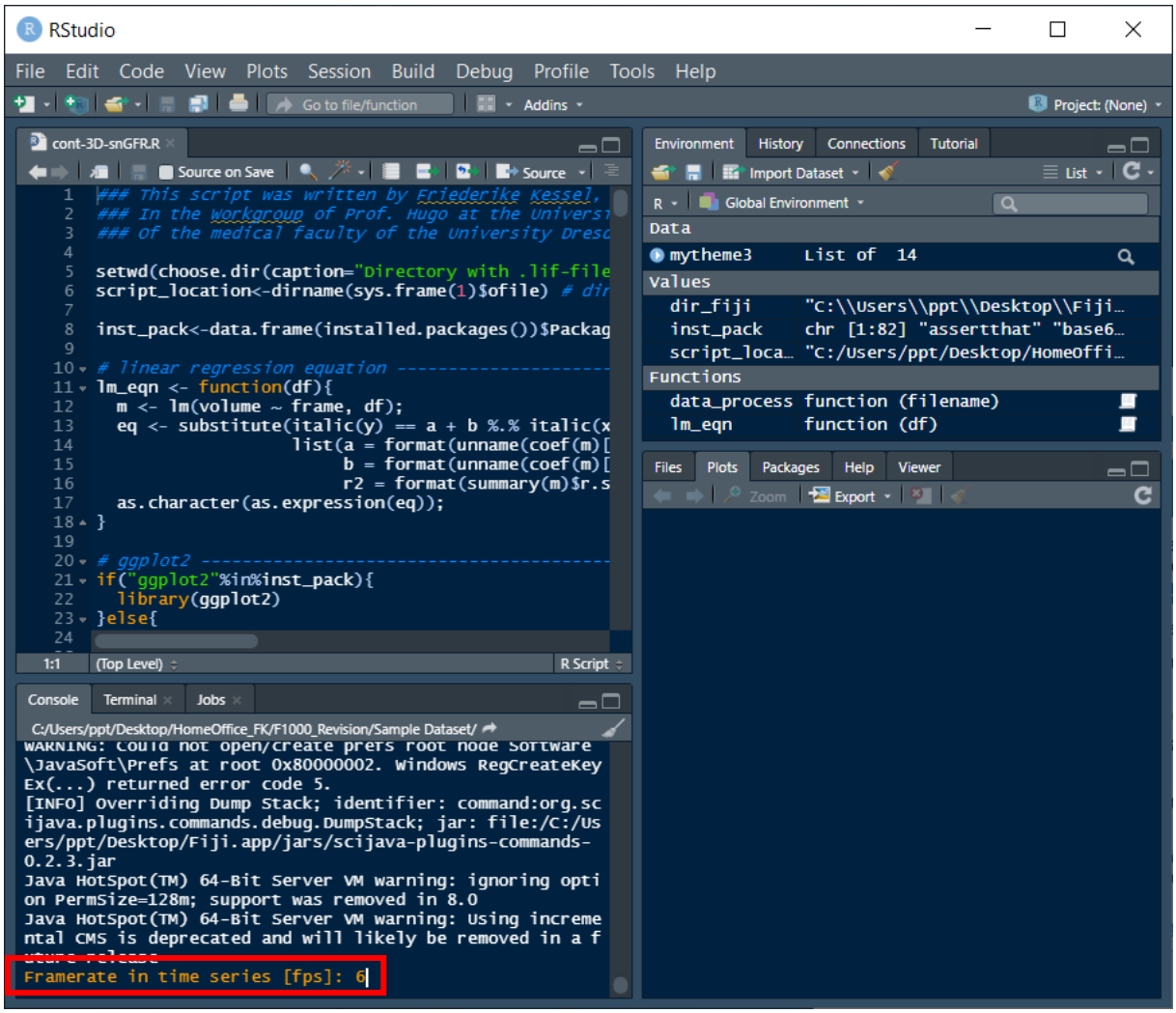



    - Confirm selection by clicking „OK“, the line ROI is saved in the „Results“ folder „{filename}_{seriesname}.zip“
    - It is necessary to select the corresponding z-stack from a list of all series in the lif file that contain the keyword PT




    - A text file containing the names, series and series numbers of the time series and the corresponding z-stacks is automatically saved „Settings_{Date}{Time}“
- The macro then processes all .lif files (this part does not require any additional manual input)
    - In every .lif file macro loops through every time series (indicated by „LY“ in name)
    - Measurement of x-y plots in time series
        - Save numerical results in txt file „{filename}_{seriesname}.txt“
    - Automatic segmentation of 3D volume
        - Save binary z-stack of segmented tubular volume „Volume_{filename}_{seriesname}.tif“
        - Alignment of line ROI to certain slice in the z-stack > saved with slice information „Volume_{filename}_{seriesname}.zip“
        - Measure cumulative volume along the length of the line ROI
            - Save numerical results in .txt file „Volume_{filename}_{seriesname}.txt“

- Subsequent automatic data analysis in R
    - Set framerate (should be consistent across all measurements)




    - The rest oft he data analysis is done automatically and does not require any additional input

## Interpreting the results

- Different intermediate results (to ensure transparency, reproducibility and to retrospectively validate the measurement and analysis)
    - Settings: Which PT belongs to which time series (in case of errors when assigning the datasets)
    - Results of x-y-plots for every time series: only needed for the data analysis
    - Results of volume measurement: only needed for data analysis
    - Selections: ROI sets (initially drawn line ROI and line ROI associated with certain slice in the z-stack)
- Binary image of segmented PT (z-stack):
    - „Volume_{filename}_{seriesname}.tif“
    - Possibility to visually check for errors in the PT segmentation (wrong segmentation results in wrong volume measurement results in wrong calculation of GFR)
- Graphs:
    - Maxima

        - „Maximum_{filename}_{seriesname}.png“
        - Plot of maximum intensity in every frame of the time series, used by the script to find the „frames of interest“ (intensity increases over background)
        - Horizontal line indicates threshold value
        - Provides information about the quality of the measurement itself
            - Double peak might indicate that the dye was injected irregularly
            - Maximum should be clearly above background
            - Peak should not be „cropped“ (indicates oversaturation of the signal)
    - Volume:
        - „Pos+Vol_{filename}_{seriesname}.png“
        - Plot of cumulative volume of the PT along the line ROI
        - Provides information about the quality of the segmentation itself
            - Irregularities might indicate errors in the automatic segmentation (check binary z-stack)
    - Intensity-Shift
        - „Cleaner_Frames_{filename}_{seriesname}.png“
        - Plot of intensity measurements in every frame (smoothed) for the frames of interest and for every position along the line ROI (converted to cumulative volume at that position)
        - Provides information about the quality of the measurement
            - Curves without a parallel shift: differences of maximum signal intensity depending on location in the PT(decreased reliability of the measurement itself)
    - Linear regression
        - „Regression_{filename}_{seriesname}.png“
        - Plot of volume shift (converted to nl) per time unit (minutes) with regression line (+confidence interval)
        - Including formula for regression line, the slope equals the GFR
        - $R^2$ as a parameter to indicate deviations from the regression line
        - Provides information about the quality of the measurement and analysis
            - Number of measurement points
            - Non-linear deviations from regression line (check raw image data: obstruction in the PT would for example lead to a decreased flow rate after a certain point, should the line ROI be adapted?)
- Result file:
    - „{Date}-Result_summary.txt“„Summary“
    - Including file name and series names
    - Median slope (median of differences between the volume shifts at each frame)
    - snGFR (actual slope of the regression line)
    - If median slope and slope of regression line (snGFR) differ clearly, this indicates that the reliability of the regression might be impaired
    - $R^2$
        - Should be above 0.9
    - Total volume of the PT segment (start and end indicated by line ROI)
        - Should be above 10 000µm³
    - Length (of line ROI)
        - Should be above 30 µm
    - Datapoints

- number of measurement points, equals number of datapoints for the regression line
- should be used to determine the reliabiliy of the measurement
- largely depends on the length of the PT segment/length of the line ROI and the framerate
- Should be > 3

## Contact information

If you need help or want to provide feedback to the workflow, don't hesitate to contact us: friederike.kessel@ukdd.de