FLUTe – (F)luorescence (L)ifetime (U)ltima(T)e (E)xplorer

a Python GUI for interactive phasor analysis of FLIM data

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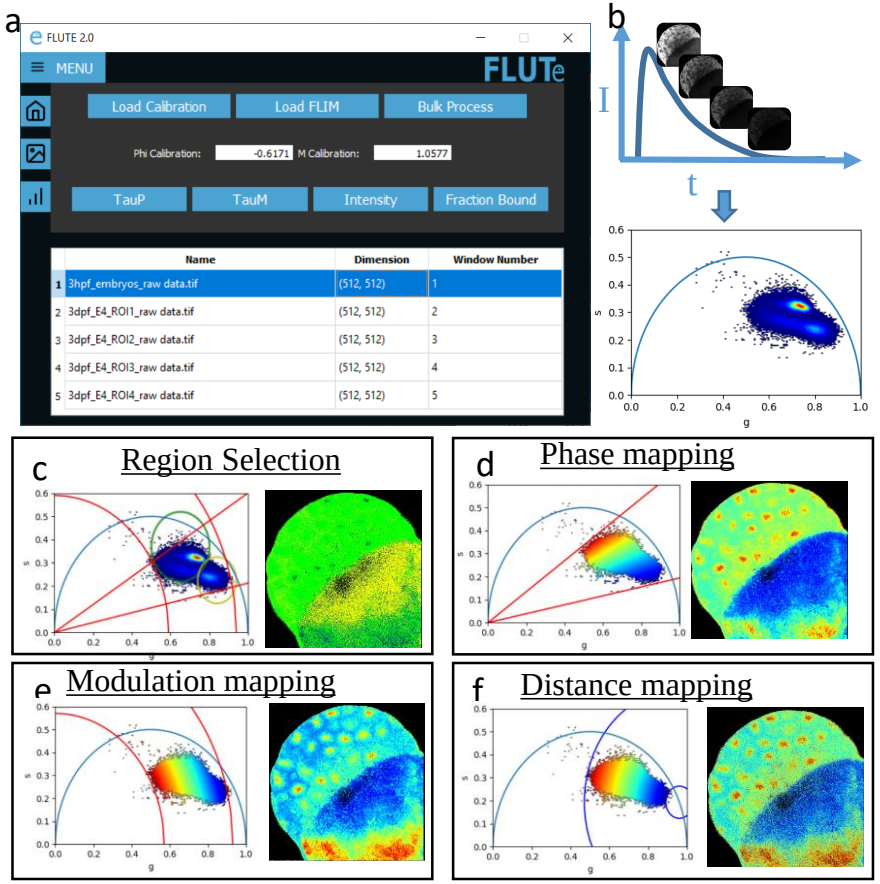
FLUTe – (F)luorescence (L)ifetime (U)ltima(T)e (E)xplorer

a Python GUI for interactive phasor analysis of FLIM data

FLUTe

- ✓ Custom written free software
- ✓ Open source code in Python
- ✓ user-friendly GUI
- ✓ large FLIM datasets



Open source code and Executable on GitHub

https://github.com/LaboratoryOpticsBiosciences/FLUTE

FLIM data in Zenodo repository

https://zenodo.org/records/8324901

Polina Kostina

Biological Imaging

Gottlieb, D., Asadipour, B., Kostina, P., Ung, T., & Stringari, C. (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. Biological Imaging, 1-22. doi:10.1017/S2633903X23000211

Data format

FLUTE performs phasor analysis on FLIM data in the time domain,

- either acquired with a time-correlated single photon counting (TCSPC) electronic cards
- time-gating technique, provided that an entire period of the laser repetition is recorded and the lifetime decay it is not truncated

FLIM data is read as a .tiff stack format

- where each image of the stack represents a temporal bin of the FLIM stack acquired in the time domain.
- FLIM data acquired with commercial cards that are not already in a .tiff format have to be first converted using either the associated commercial software or available open-source plugins (See Supplementary Information 11).

Becker & Hickl

Conversion of *Becker* & Hickl (.sdt) files to .tiff files
.std files from *Becker* & Hickl can be exported into .tiff files using the opensource plugin *Bio-Formats toolbox*

ISS

Conversion of ISS (.fbd) files to .tiff files
.fbd files from ISS can be exported into .tiff files from The VistaVision software (See also *VistaVision* manual pages 28-29)

PicoQuant

Conversion of Picoquant (.ptu) files to .tiff files
.ptu files from *Picoquant* can be exported into .tif files using first SymPhoTime64tware software to extract the binary .bin file and then files using the opensource plugin *Bio-Formats toolbox* to convert the .bin file in .tif
Alternatively .ptu files from Picoquant can be exported directly into .tiff files using the open source plugins PTU_Reader for Image J (*https://github.com/UU-cellbiology/PTU_Reader*.)

Leica

Conversion to .bin files
Then transformed to tif files with *Bio-Formats toolbox*

Free and open source software to perform phasor analysis of FLIM data

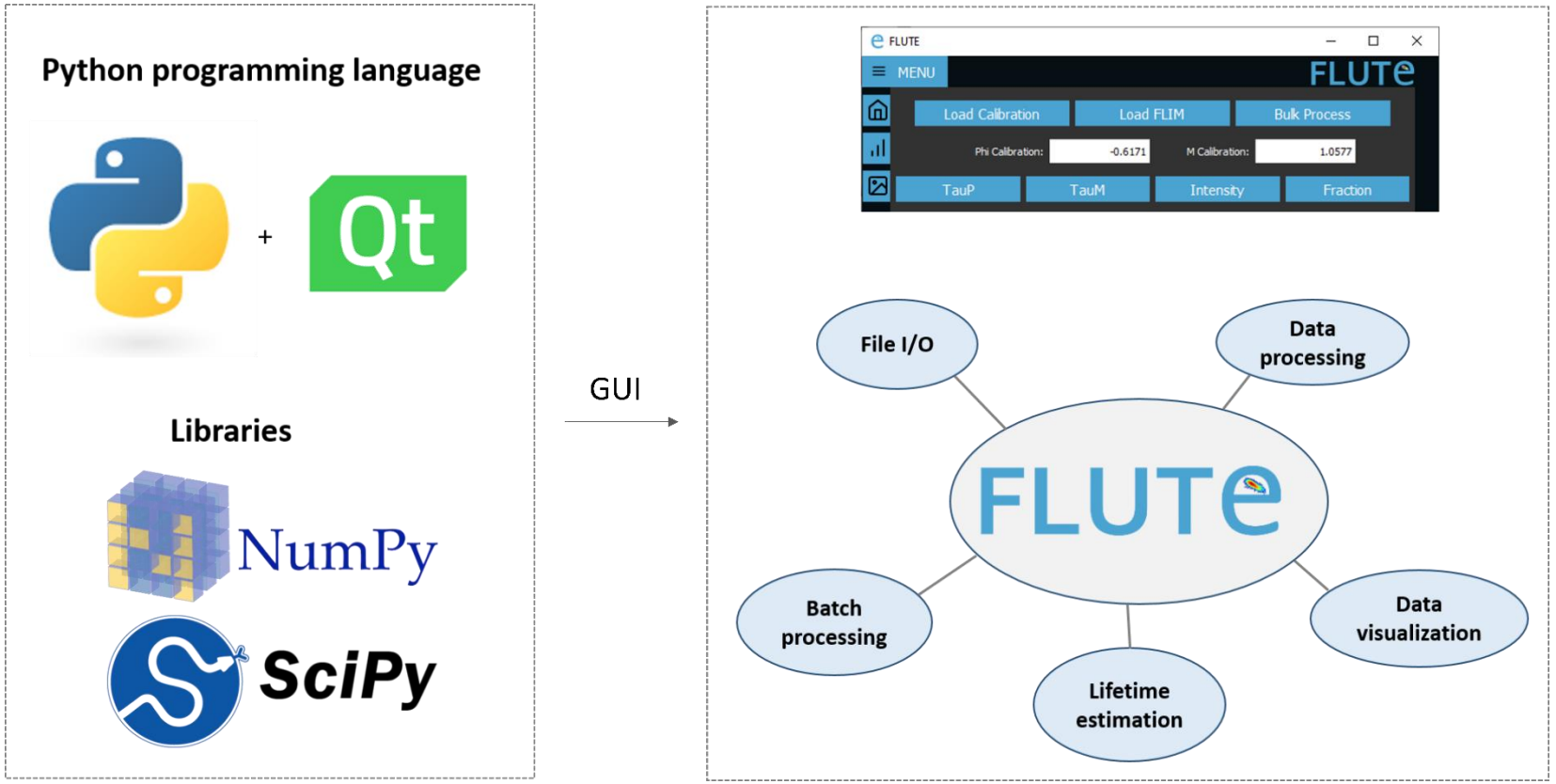
Reproducibility
Open access
Easy and quantitative analysis

Software	SimFCS	PAM	FLIMJ	FLUTE
Relative publication	Ranjit et al. 2018	Schrimpf et al. 2018	Gao et al. 2020	
Open source code	No	Yes	Yes	**Yes**
Free	Yes	No	Yes	**Yes**
Programming language	C++	MATLAB	Java	**Python (facilitates broad use and extensibility)**

Phyton

- Napari FLIM phasor plotter (C. Wetzker)
- Phasor Identifier: A Cloud-based Analysis of Phasor-FLIM Data on Python Notebooks (F. Cardarelli)

GUI Development and Implementation

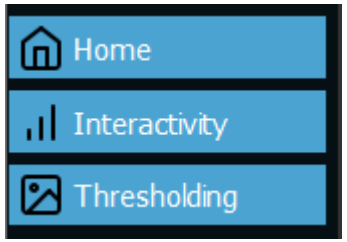


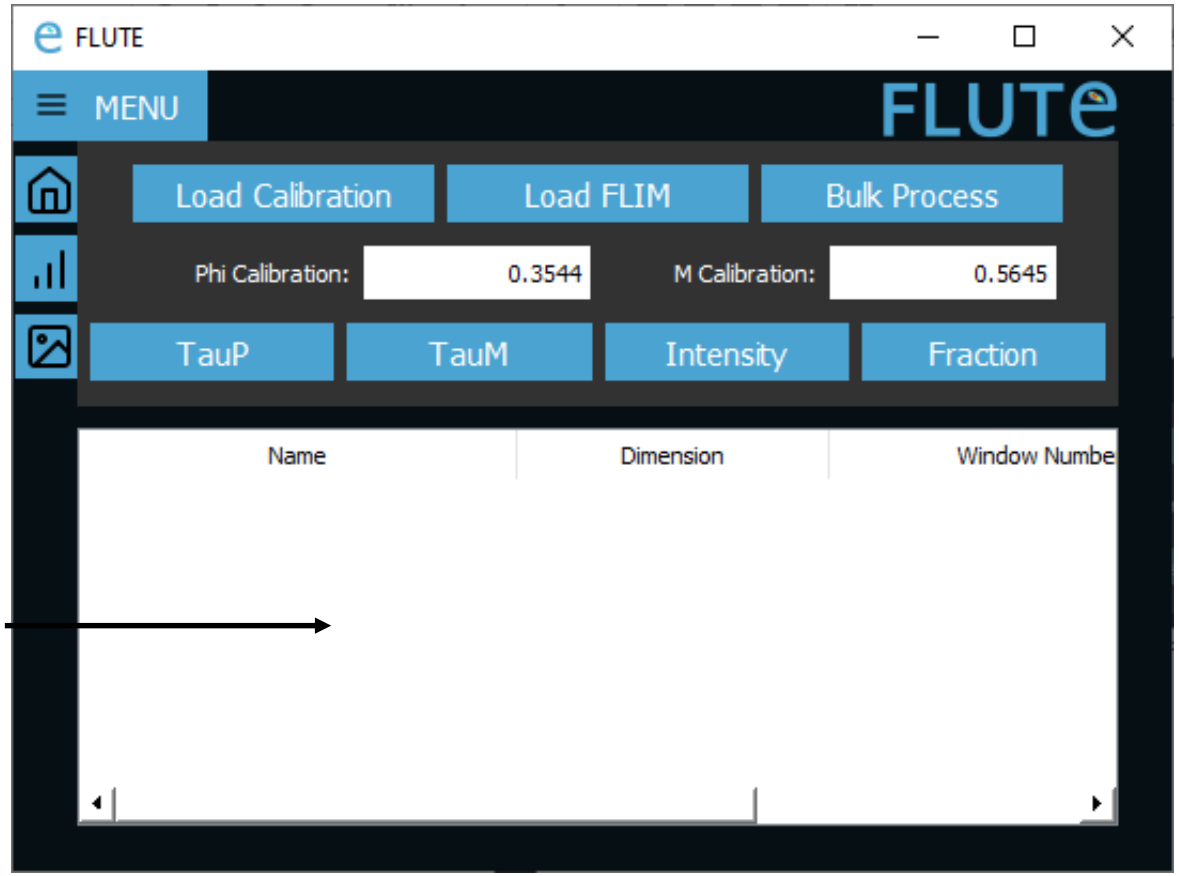
- **Run 'main.py'** in Pyhton after installing all the necessary packages (PyQt5, numpy, opencv-python, matplotlib, scikit-image). Works in OS: Windows, Linux and MacOS including M1 and M2 chips.

- **Use the GUI 'FLUTE.exe'**

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

Menu button





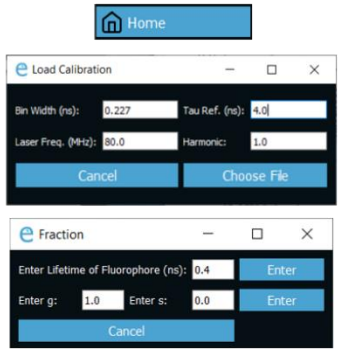
Table

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

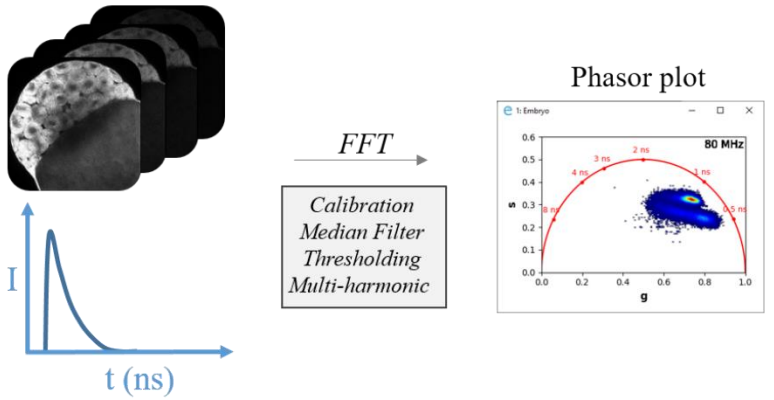
Data Processing

.tiff

a **Input parameters**

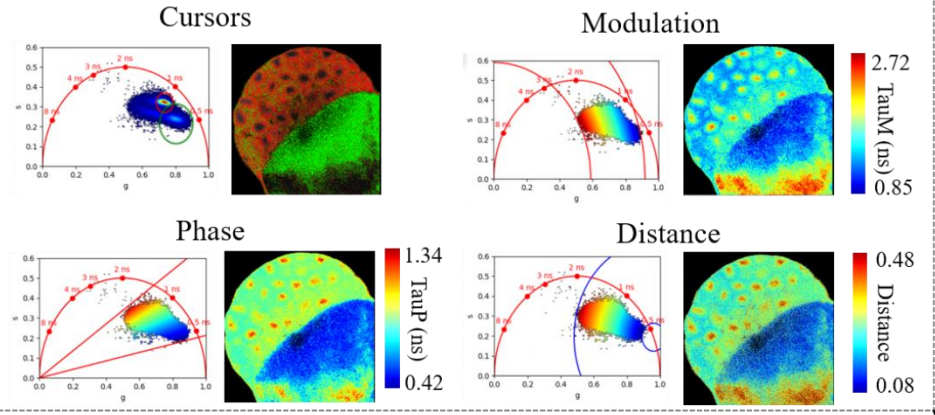


b Raw FLIM data **Data processing**



c **Filters and visualization tools**

d **Visualization and lifetime estimation**

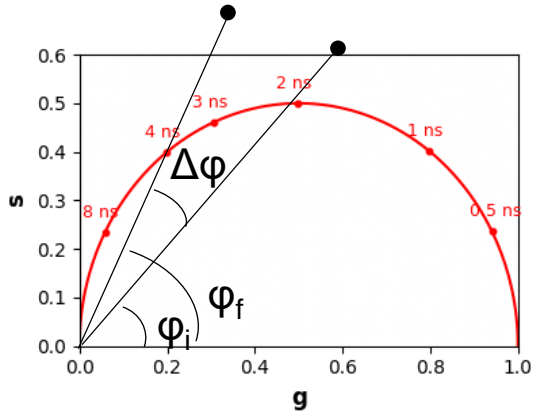


Processing times with a MacBook Air (M1, 2020, 8 GB Memory) for a phasor transformation of FLIM stacks are: ~0.7 s for a 256 x 256 images, ~1.1 s for a 512x512 images and ~3 s for a 1024 x 1024 images with 56 time beans each

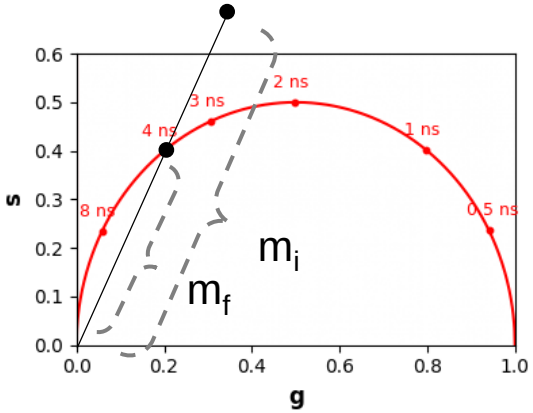
Save results .txt .tiff

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

b. Calibration with reference lifetime

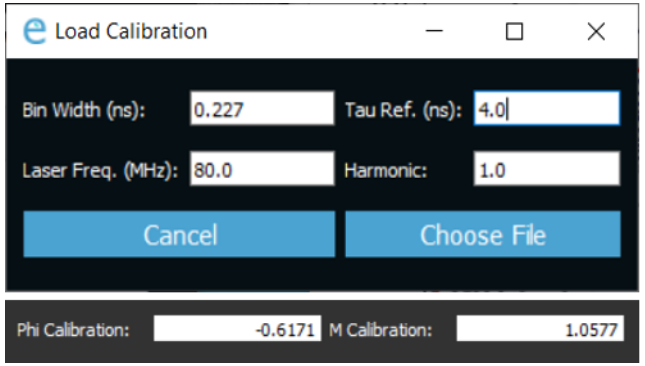


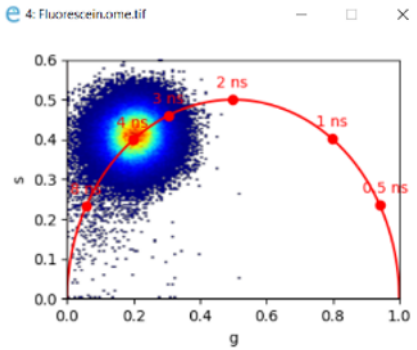
$$\varphi_f = \varphi_i + \Delta\varphi$$



$$m_f = m_i * \Delta m$$

Calibration with fluorophores with a known lifetime, *e.g.* Fluorescein (4 ns), Coumarin 6 (2.5 ns), Rodamine B (1.74 ns), or Rose Bengal (0.52 ns), or 0 ns lifetime of SHG signal from starch or KTP NPs

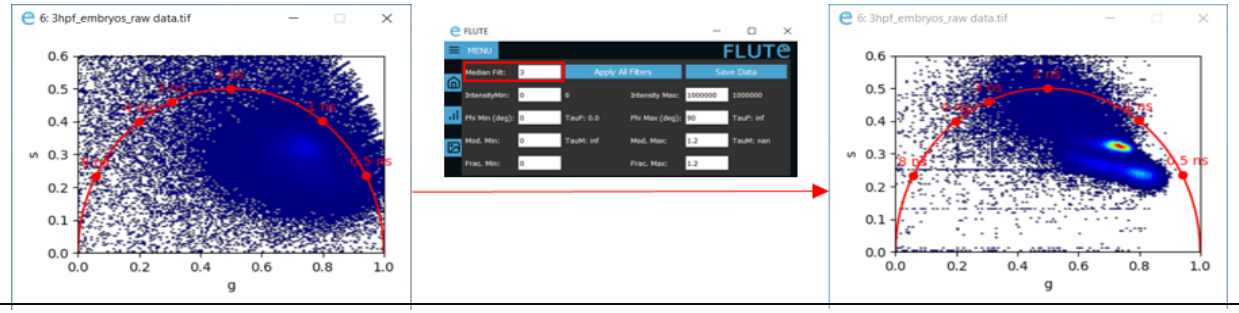




- **Bin Width (ns):** Duration of a single temporal bin of the time-domain FLIM acquisition
- **Laser Freq. (MHz):** Laser repetition rate
- **Tau Ref. (ns):** Known lifetime of the single-exponential reference sample
- **Harmonic**: Integer multiple applied to calculate the Fourier transform

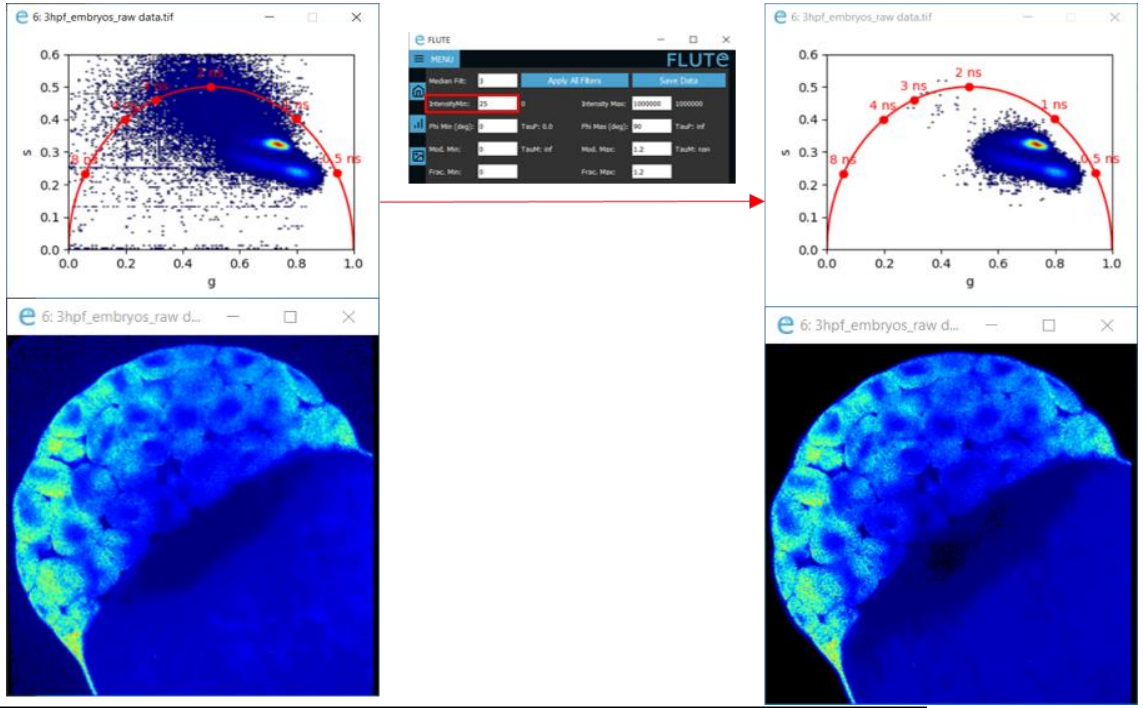
Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

c. Median filter



A 3x3 convolutional median filter is applied n times to the phasor plot

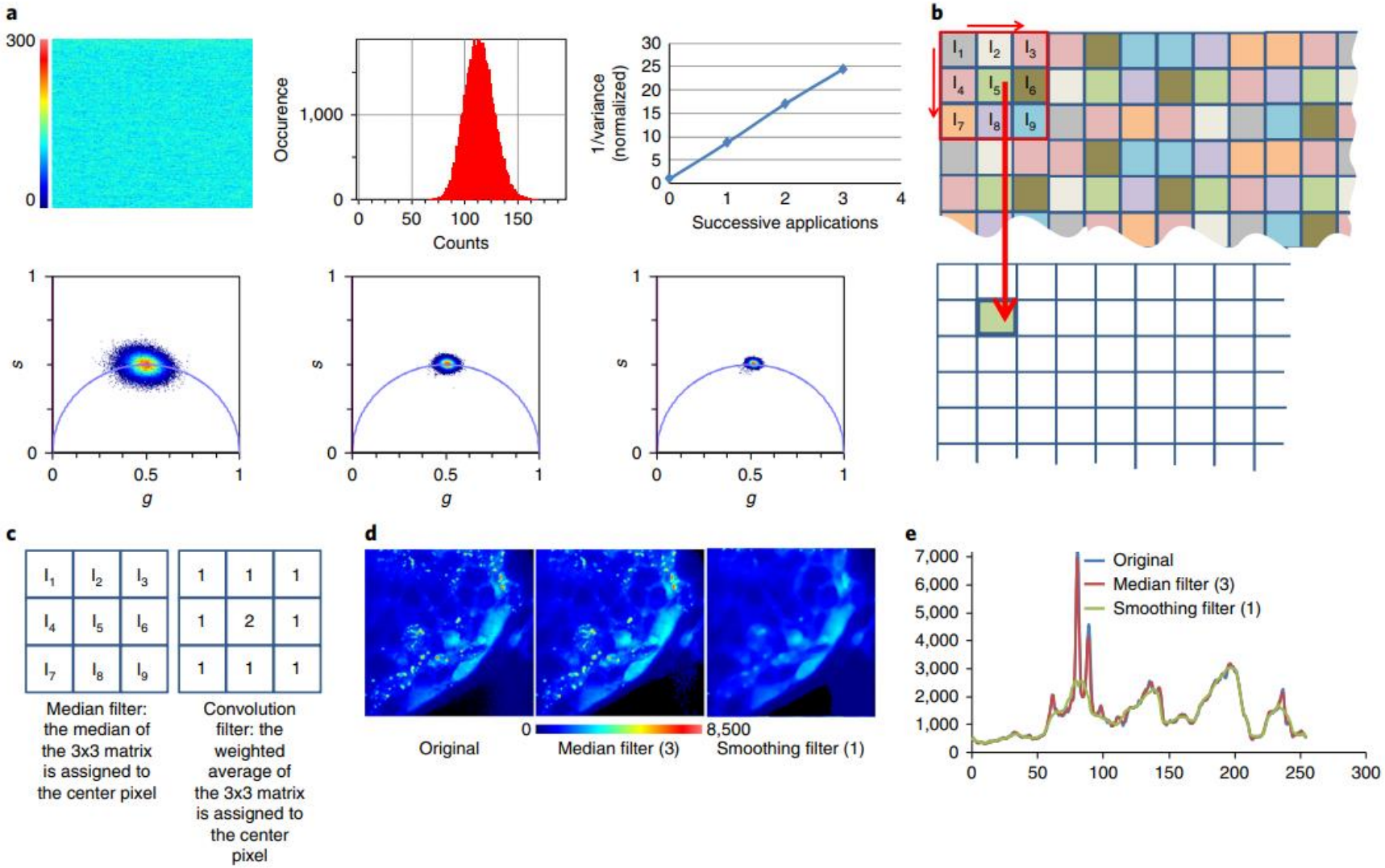
d. Intensity threshold



Changing the max and min of the intensity threshold

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

Median filter



Ranjit, S., et al., Fit-free analysis of fluorescence lifetime imaging data using the phasor approach. Nat Protoc, 2018.

Lifetime estimation and data visualization

a. Interactive exploration of FLIM data

Interactivity window
- Selection of Cluster with different size and colors
- Interactive adjustment of the colour map thresholds

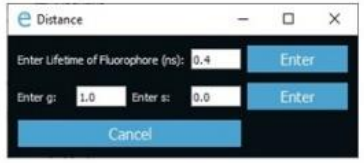
Thresholding window
- Fine adjustment of the colour map thresholds

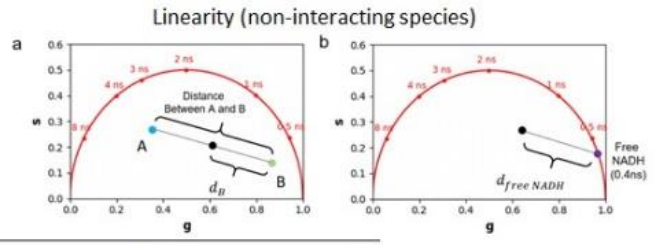
b. Lifetime estimation

Phase Lifetime $\tau_\phi = \frac{1}{\omega}\tan(\varphi) = \frac{1}{\omega}\frac{s}{g}$

Modulation Lifetime $\tau_m = \frac{1}{\omega}\sqrt{\frac{1}{m^2}-1} = \frac{1}{\omega}\sqrt{\left(\frac{1}{s^2+g^2}-1\right)}$

Distance from known molecular species (i.e. free NADH)

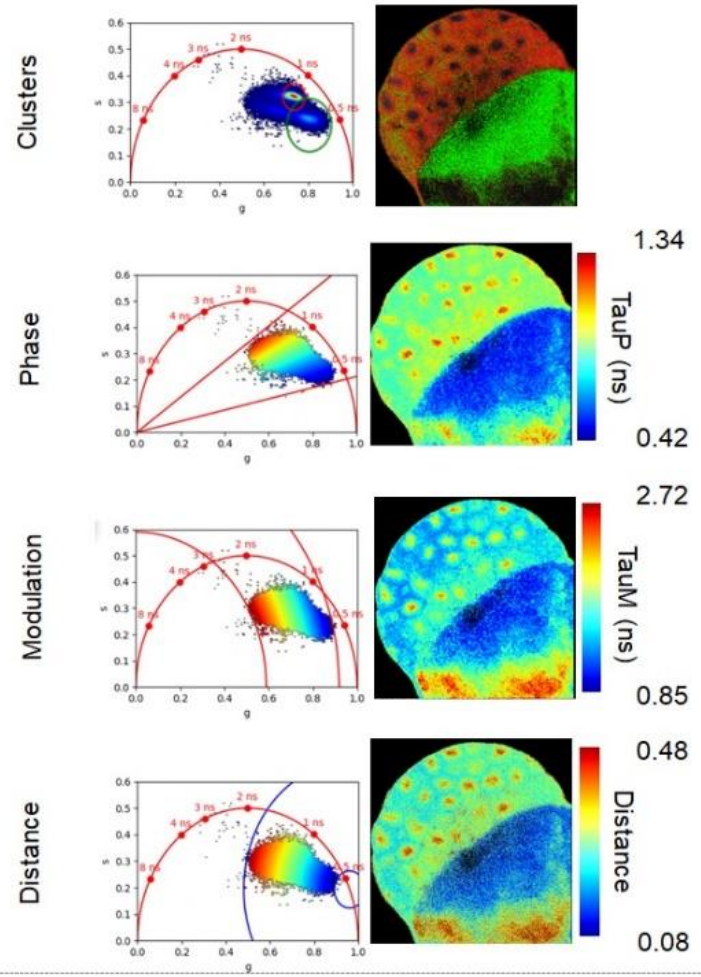




$$d_{NADH} = \sqrt{\left(g_{exp} - g_{fNADH}\right)^2 + \left(s_{exp} - s_{fNADH}\right)^2}$$

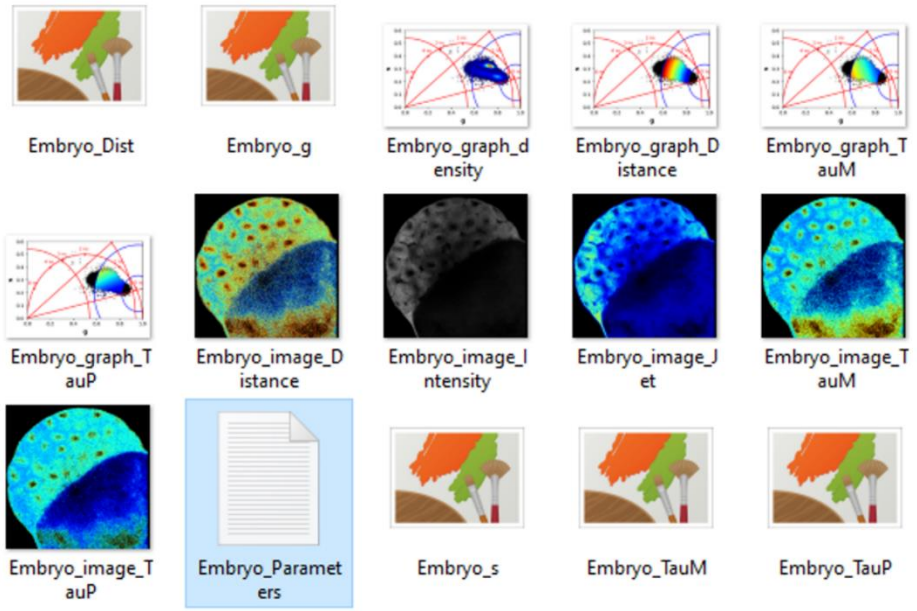
c. Image and phasor visualization options

- User-friendly switch between contrasts
- Different lifetime contrasts
- Simultaneous mapping of the FLIM image and of the phasor plot
- clear representation and an interactive exploration of the FLIM data



Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

Exporting results and batch processing



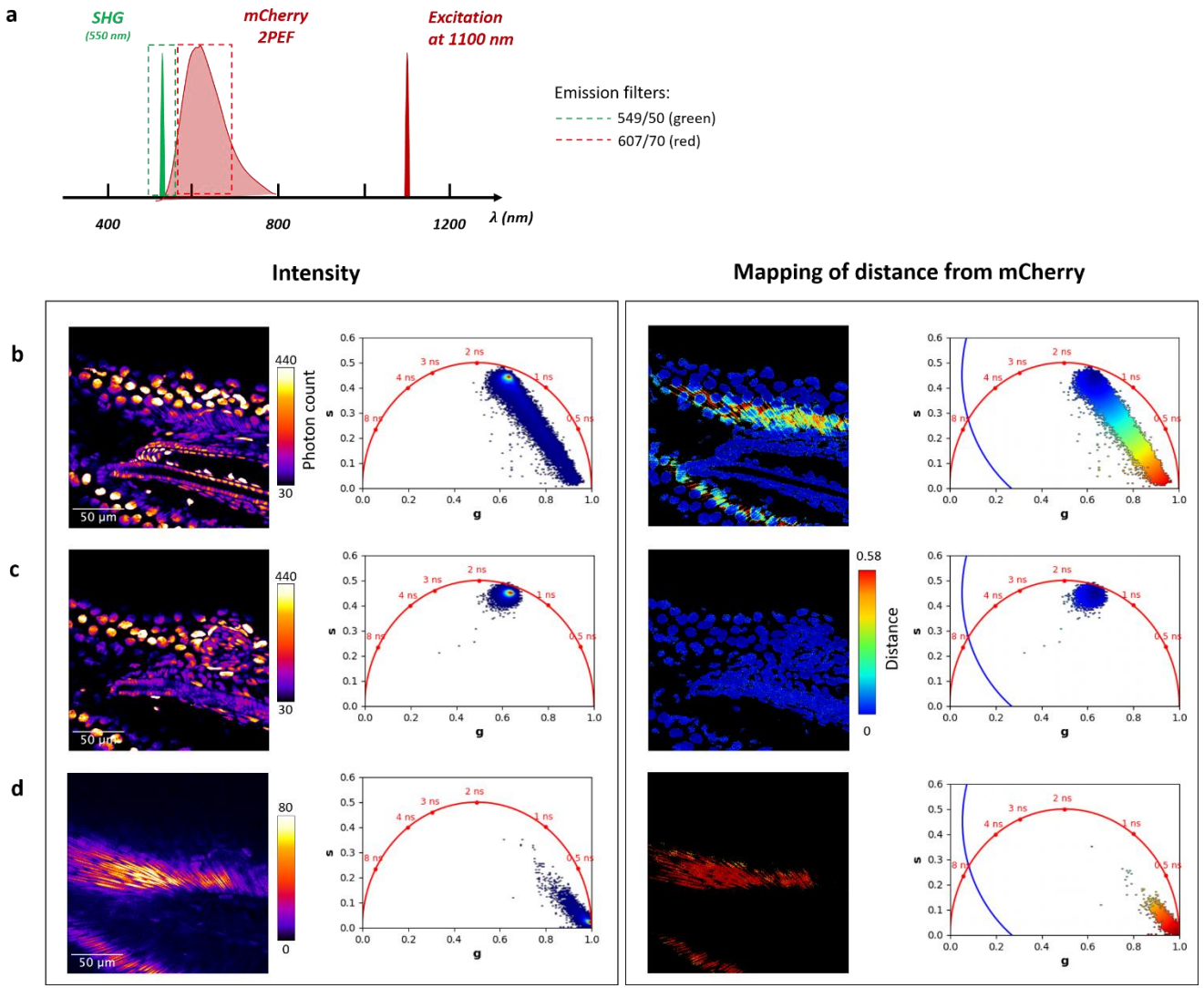

```
Intensity Min: 25.000
Intensity Max: 1000000.000
Phi Min (Deg, ns): (18.000, 0.646)
Phi Max (Deg, ns): (64.000, 4.079)
Modulation Min (M, ns): (0.540, 3.101)
Modulation Max (M, ns): (0.940, 0.722)
Distance From Coordinates (g,s): 0.961, 0.193
Distance Min: 0.260
Distance Max: 0.900


Average g Coordinate: 0.687
Average s Coordinate: 0.307
Average TauP (ns): 0.891
Average TauM (ns): 1.744
Average distance: 0.327
```

Saving results.
Saved FLIM images and phasor plots (left) and applied filters (right) to create the mask and measurements of the average of g, s, TauPhase (TauP), TauModulation (TauM) and distance (right).

Batch processing on multiple FLIM images
Using the same parameters
Typical processing times for the full analysis of one image (that includes phasor transformation, applications of filters, saving results, images and measurements) with a MacBook Air (M1, 2020, 8 GB Memory) are:
~1.8 s for a 256 x 256 image
~2.5 s for a 512x512 image
~5.4 s for a 1024 x 1024 image

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

a. Mapping of distance from mCherry in a 5-day post-fertilization (dpf) zebrafish embryo tail (H2B-mCherry line) *in vivo.*



FLIM data in Zenodo repository

https://zenodo.org/records/8324901

- temporal bin number = 56
- laser repetition rates = 80 MHz
- bin width = 0.223ns

starch SHG-IRF.tif stack contains the measurement of the SHG signal from starch, with a known lifetime of 0ns, used as calibration for ZF-1100_noEF.tif, ZF-1100_607-70_filter.tif and ZF-1100_550-49 _filter.tif stacks

ZF-1100_noEF.tif contains the fluorescence intensity decay of the zebrafish embryo at 5 days post fertilization acquired without emission. This file needs to be calibrated with starch SHG-IRF.tif stack.

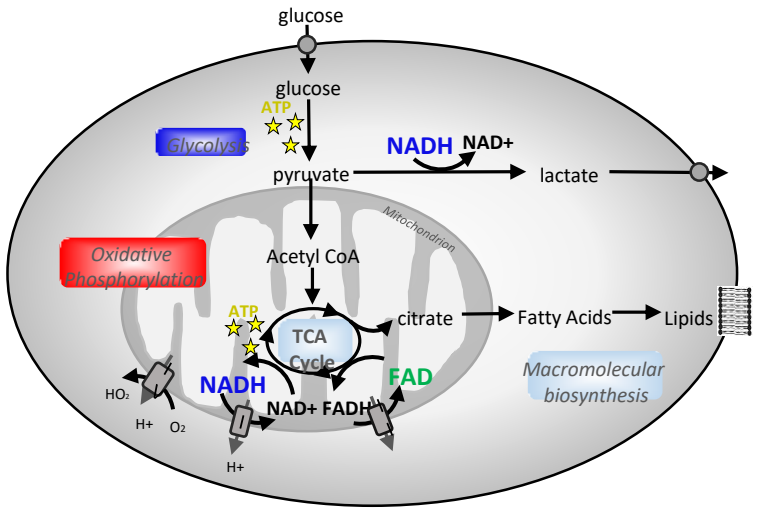
ZF-1100_607-70_filter.tif contains the fluorescence intensity decay of the zebrafish embryo at 5 days post fertilization acquired with an emission filter a 607/70 nm. This file needs to be calibrated with starch SHG-IRF.tif stack

ZF-1100_550-49 _filter.tif contains the fluorescence intensity decay of the zebrafish embryo at 5 days post fertilization acquired with an emission filter of 549/50 nm. This file needs to be calibrated with starch SHG-IRF.tif stack.

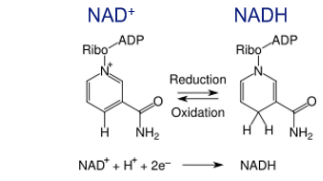
Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

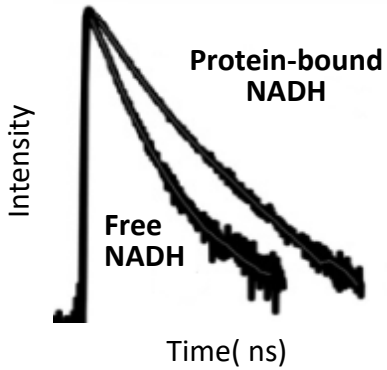
FLIM and intrinsic **biomarker NADH for metabolic imaging**

Nicotinamide adenine dinucleotide (NADH)

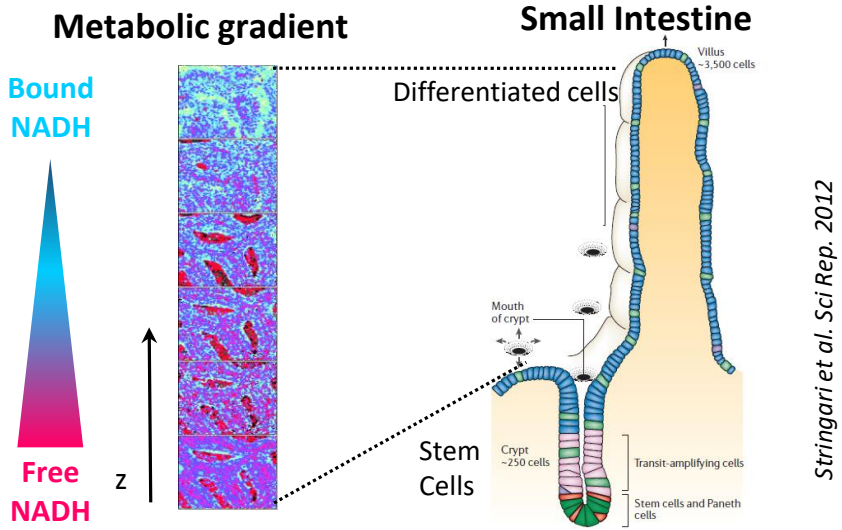


Stringari et al Sci Rep. 2017





Lakowicz et al.. PNAS. 1992
Heikal at al. Biomark Med. 2010
Stringari et al. PNAS. 2011

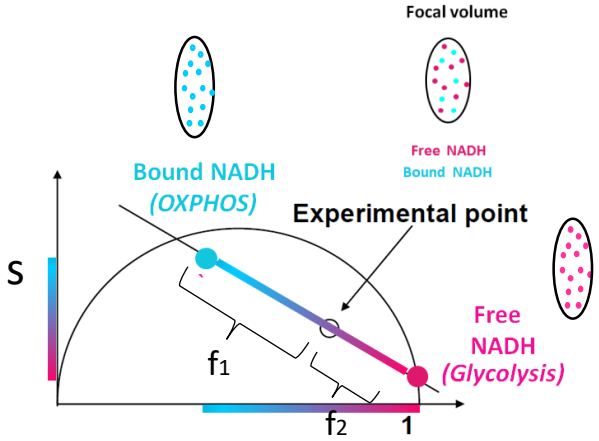


Stringari et al. Sci Rep. 2012

Resting cell → **Proliferating cell**

OxPhos ++
Long NADH Lifetime
Bound NADH

Glycolysis ++
Short NADH Lifetime
Free NADH

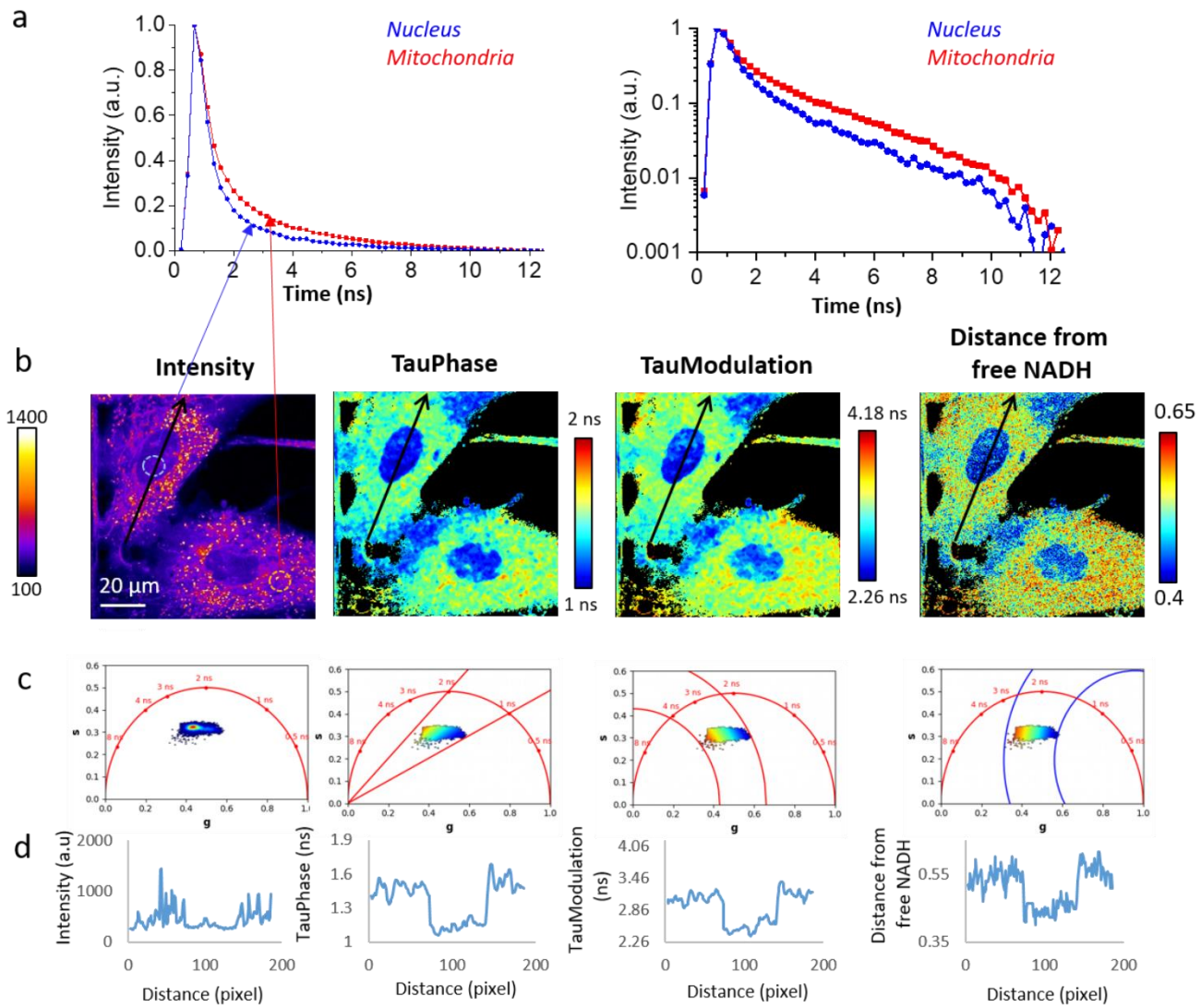


Stringari et al PNAS 2011
Stringari et al Sci Rep. 2012

fB NADH $\propto \frac{NAD+}{NADH}$

Stringari et al. Sci Rep. 2017
Ung et al et al. Sci Rep. 2021
Sánchez-Ramírez, et al. Journal of Cell Biology 2022
Paillon et al . Molecular Biology of the Cell 2024
Sánchez-Ramírez, et al. Mol Neurobiol. 2024

b. FLIM analysis of NADH reveals intracellular metabolic heterogeneity in mesenchymal stromal cells.



FLIM data in Zenodo repository

https://zenodo.org/records/8324901

- temporal bin number = 56
- laser repetition rates = 80 MHz
- bin width = 0.223ns

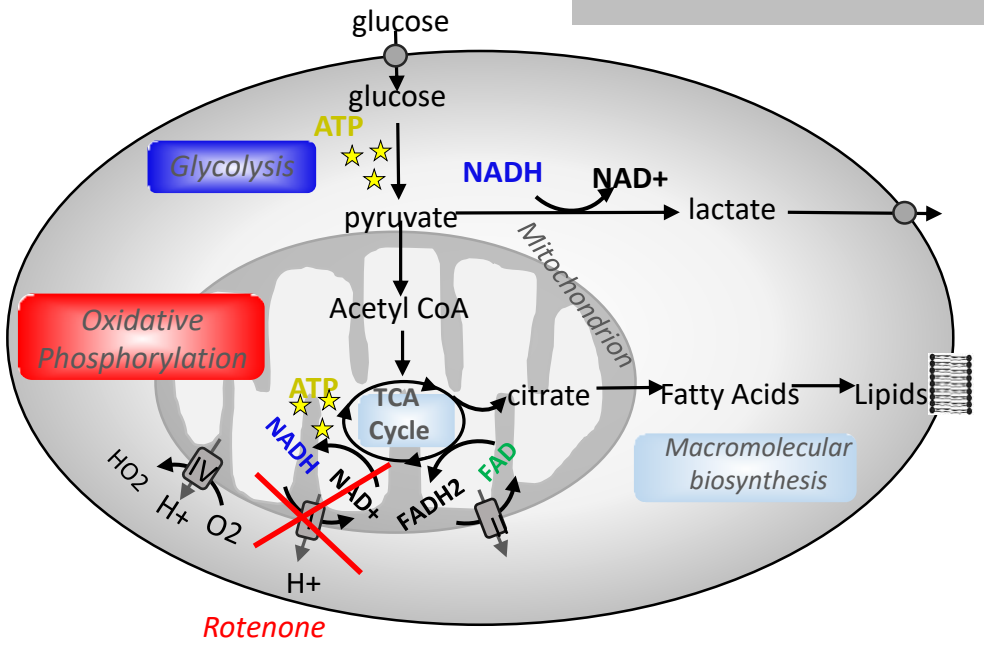
Fluorescein_hMSC.tif stack contains the fluorescence intensity decay of fluorescein solution with a known lifetime of 4ns, used as calibration for the hMSC-ZOOM, hMSC control, hMSC_rotenone stacks.

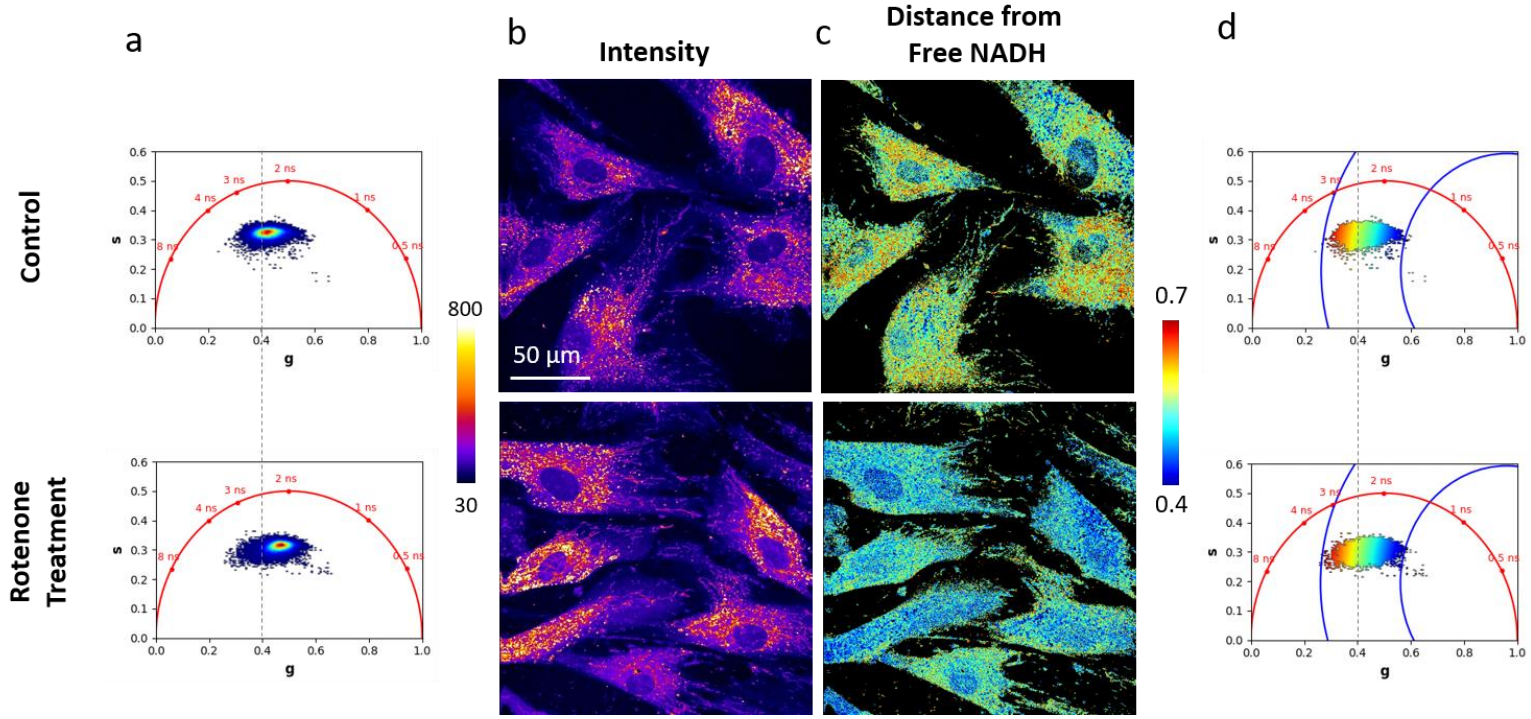
hMSC-ZOOM.tif and ***hMSC control.tif*** stacks contain the fluorescence intensity decay of mesenchymal stromal cells in control condition. This files need to be calibrated with Fluorescein_hMSC.tif stack

hMSC_rotenone.tif stack contains the fluorescence intensity decay of mesenchymal stromal cells treated with rotenone. This file needs to be calibrated with Fluorescein_hMSC.tif stack

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

c. Shift in the distance from free NADH in live cells upon metabolic treatment.





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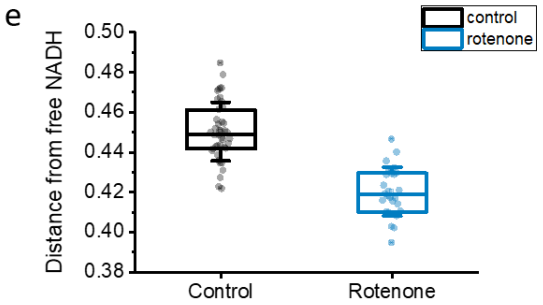

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

Future Developments

- **Direct import of the common FLIM file formats** (.std, .fbd and .ptu) inside the Python code by using already available Python libraries and open-source codes
- **Intermediary file format** which encompasses matrices for Intensity, g and s coordinates.
- **Increase FLUTE speed** with parallel computing capabilities of Graphics processing units (GPUs) by using specialized Python libraries such as Numba and CuPy for real time phasor analysis and representation during experiments.
- **Adapt phasor analysis to typical time-gated sampling limitations** by taking in account the effect of decay truncation and gate shape
- Integrate a **fully automated calculation and mapping of fraction of molecules** (with 2 known moleculr species)
- **Advanced analysis tools** such as, different filters, freehand cluster drawing, cluster analysis with Machine Learning, FRET trajectory estimation and calculation of absolute concentration of NADH.
- Napari

Gottlieb et al (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. *Biological Imaging, 1-22.*

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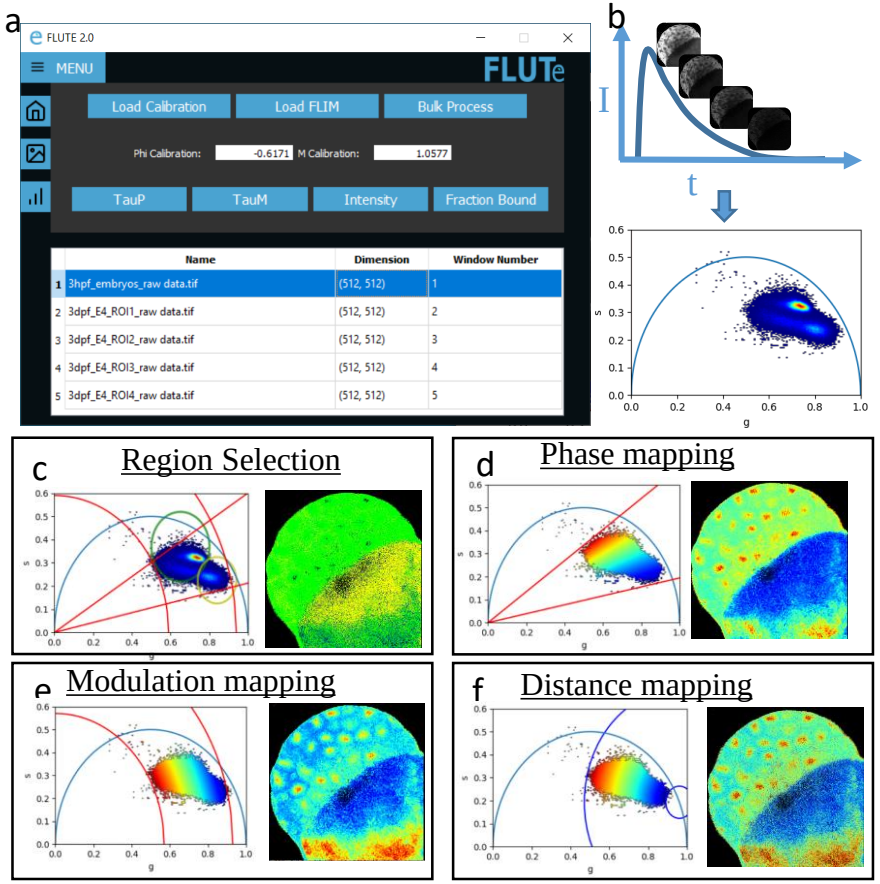

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Polina Kostina

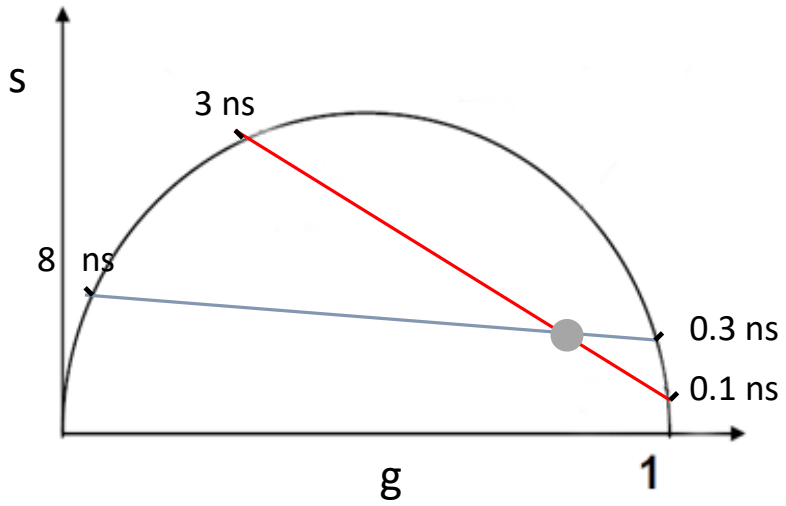
Gottlieb, D., Asadipour, B., Kostina, P., Ung, T., & Stringari, C. (2023). FLUTE: A Python GUI for interactive phasor analysis of FLIM data. Biological Imaging, 1-22. doi:10.1017/S2633903X23000211

Multi harmonic phasor analysis

Multi-harmonics analysis separates different molecular components that have the same location in the phasor plot at one harmonic but arise from different lifetime distributions.

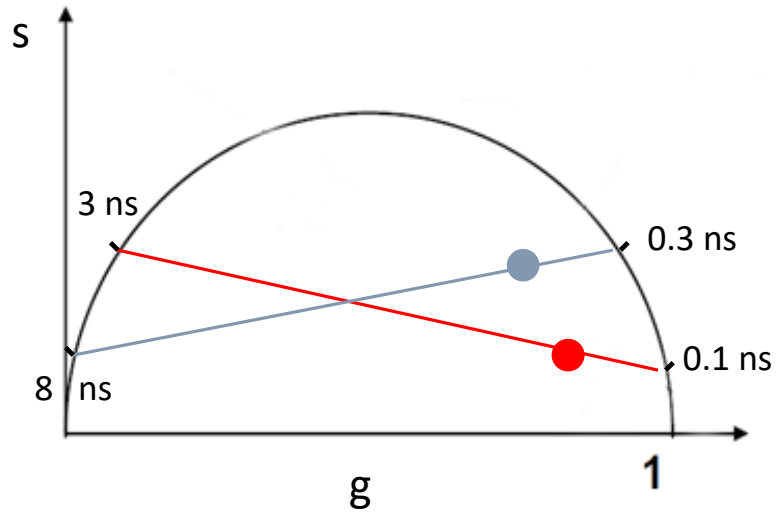
1st harmonic

$\omega = \omega_0$



2nd harmonic

$\omega = 2\ \omega_0$



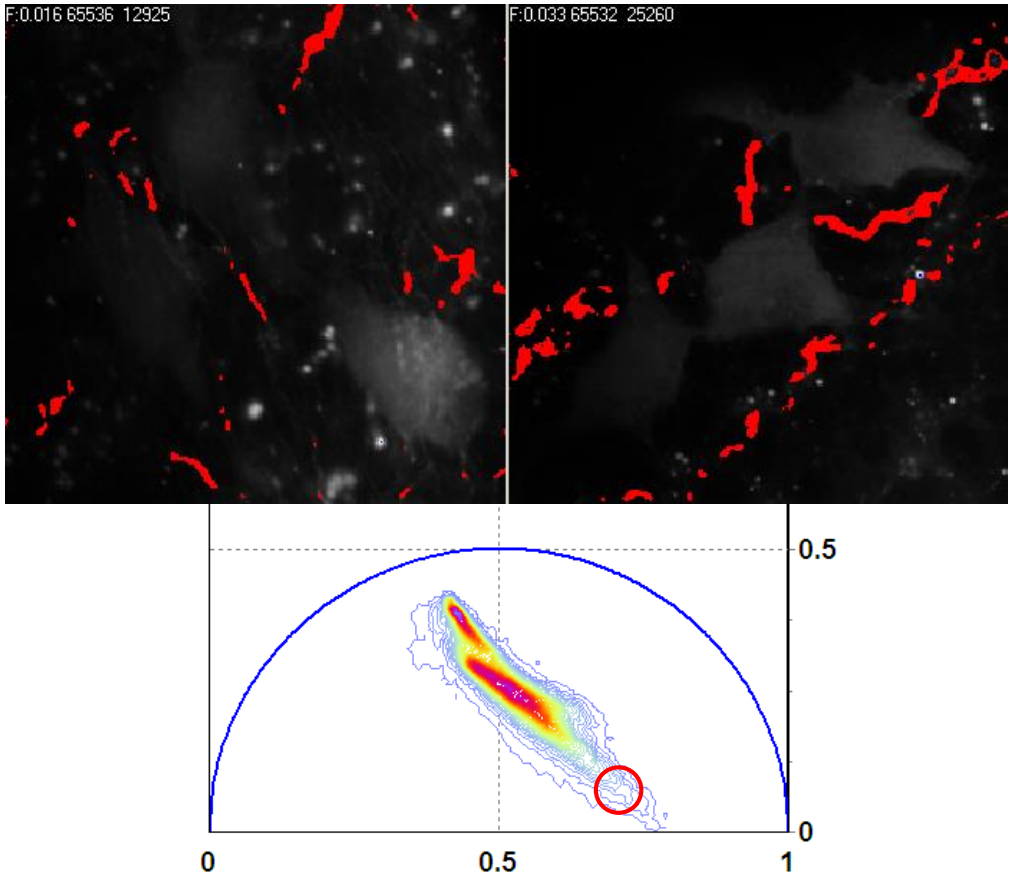
f = 80 MHz

Stringari et al. *PNAS* 2011

Multi harmonic analysis

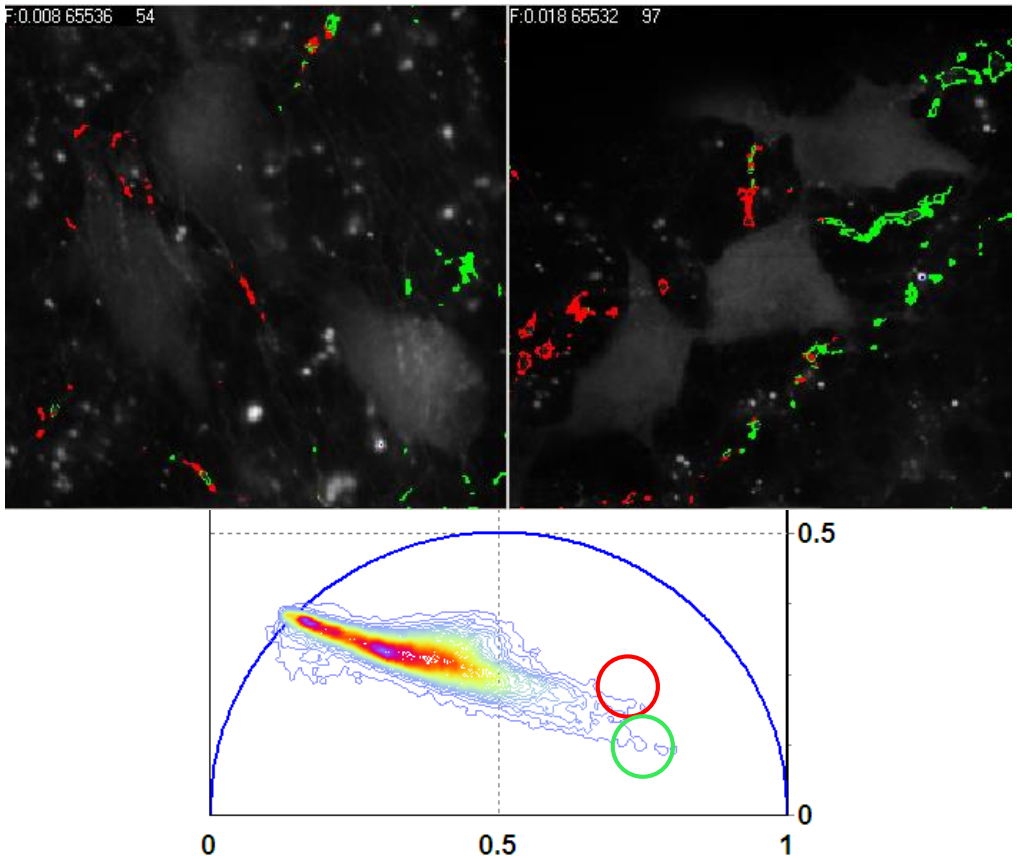
1st harmonic FFT

$\omega = \omega_0$



2nd harmonic FFT

$\omega = 2\ \omega_0$



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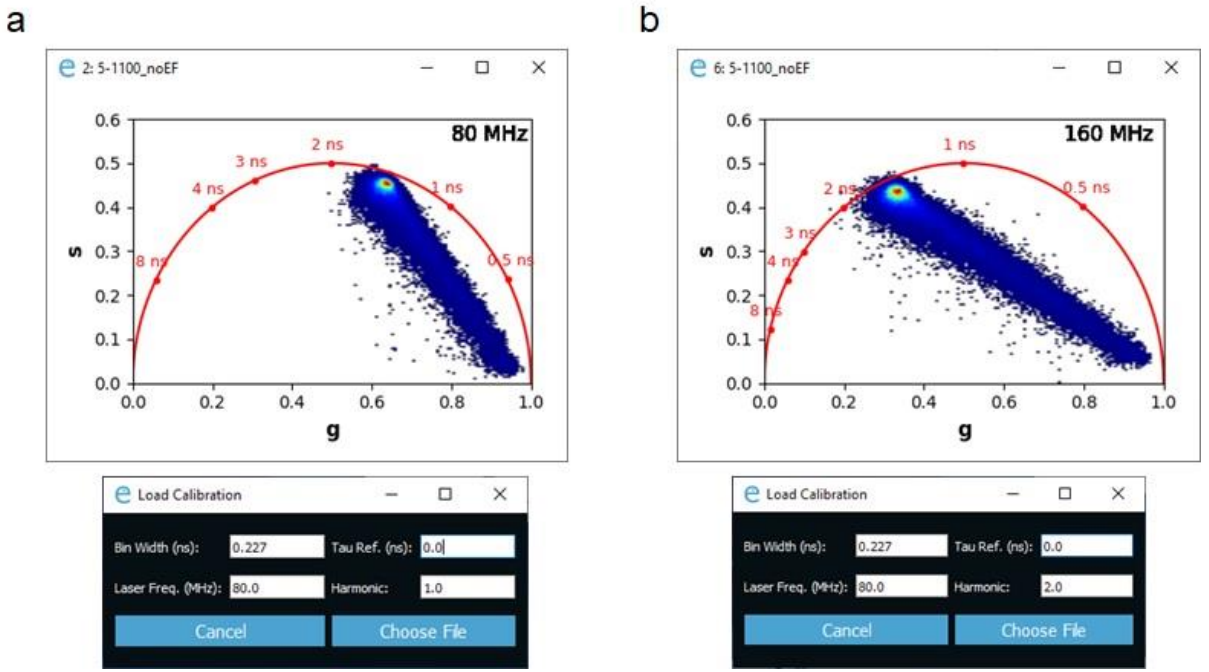

Figure S5: *Calibration of* ***FLIM image (ZF-1100_noEF.tiff) using the SHG at 0 ns lifetime (starch SHG-IRF.tiff)*** *as a calibration. Phasor transformation and plot are calculated at the first (A) and second (B) harmonic of the laser repetition rate.*

FLIM data in Zenodo repository

https://zenodo.org/records/8324901

All files have been acquired with the following parameters:

- temporal bin number = 56
- laser repetition rates = 80 MHz
- bin width = 0.223ns

Fluorescein_embyo.tif stack contains the fluorescence intensity decay of fluorescein solution with a known lifetime of 4ns, used as calibration for the Embryo.tif stack

Embryo.tif file contains the fluorescence intensity decay of a zebrafish embryo at 3 days post fertilisation. This file needs to be calibrated with Fluorescein_embyo.tif stack

Fluorescein_hMSC.tif stack contains the fluorescence intensity decay of fluorescein solution with a known lifetime of 4ns, used as calibration for the hMSC-ZOOM, hMSC control, hMSC_rotenone stacks.

hMSC-ZOOM.tif and ***hMSC control.tif*** stacks contain the fluorescence intensity decay of mesenchymal stromal cells in control condition. This files need to be calibrated with Fluorescein_hMSC.tif stack

hMSC_rotenone.tif stack contains the fluorescence intensity decay of mesenchymal stromal cells treated with rotenone. This file needs to be calibrated with Fluorescein_hMSC.tif stack

starch SHG-IRF.tif stack contains the measurement of the SHG signal from starch, with a known lifetime of 0ns, used as calibration for ZF-1100_noEF.tif, ZF-1100_607-70_filter.tif and ZF-1100_550-49 _filter.tif stacks

ZF-1100_noEF.tif contains the fluorescence intensity decay of the zebrafish embryo at 5 days post fertilization acquired without emission. This file needs to be calibrated with starch SHG-IRF.tif stack.

ZF-1100_607-70_filter.tif contains the fluorescence intensity decay of the zebrafish embryo at 5 days post fertilization acquired with an emission filter a 607/70 nm. This file needs to be calibrated with starch SHG-IRF.tif stack

ZF-1100_550-49 _filter.tif contains the fluorescence intensity decay of the zebrafish embryo at 5 days post fertilization acquired with an emission filter of 549/50 nm. This file needs to be calibrated with starch SHG-IRF.tif stack.