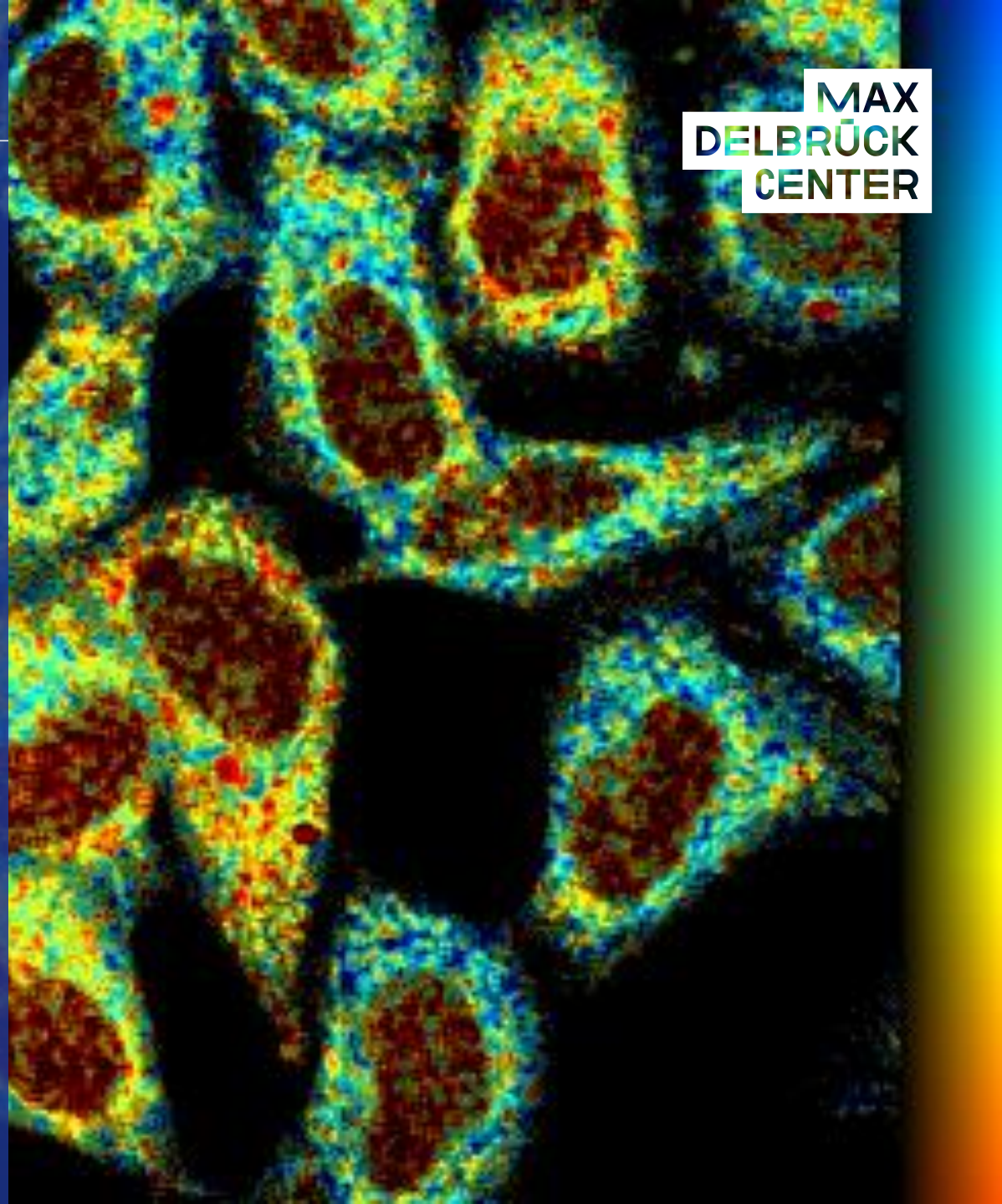
ANALYSING DATA WITH *FLIMfit*



Anca Margineanu
Advanced Light Microscopy

FLIMfit: FITTING ALGORITHMS

- **Publication: Warren** et al. (2013), *Rapid Global Fitting of Large Fluorescence Lifetime Imaging Microscopy Datasets,* PLoS ONE 8(8): e70687. doi:10.1371/journal.pone.0070687
- **Website**: https://flimfit.org/
- Developed in **Matlab,** offered as a **compiled GUI** that runs independently (only the **MCR** necessary) to analyse **time-domain FLIM** data
- Several versions are implemented in **Omero**

- **Global fitting algorithm** for **multiexponential decays** applied on **hundreds of images**

$$y(t) = \beta_1 e^{-t/\tau_1} + \beta_2\, e^{-t/\tau_2}$$

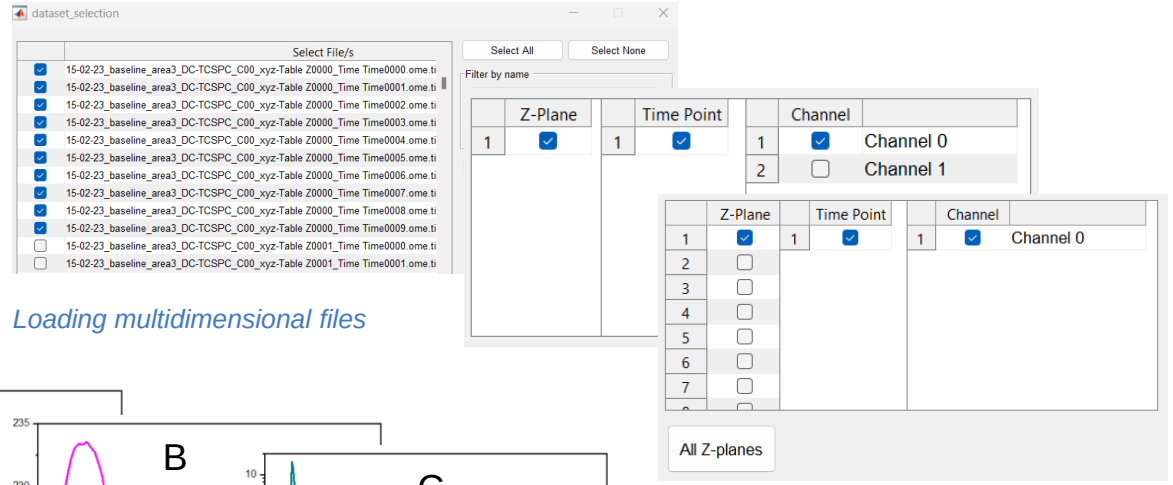
  - **Global analysis**: lifetimes are considered invariant across all images or regions of interest and are fitted simultaneously by minimising a global χ^2

tau_1	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03	1.8588e+03
tau_2	166.7117	166.7117	166.7117	166.7117	166.7117	166.7117	166.7117	166.7117	166.7117	166.7117
offset	0.0563	0.0446	0.0438	0.0456	0.0497	0.0393	0.0484	0.0499	0.0438	0.0449
beta_1	0.2558	0.2465	0.2308	0.2296	0.2218	0.2082	0.2155	0.2040	0.2007	0.2089
beta_2	0.7442	0.7535	0.7692	0.7704	0.7782	0.7918	0.7845	0.7960	0.7993	0.7911

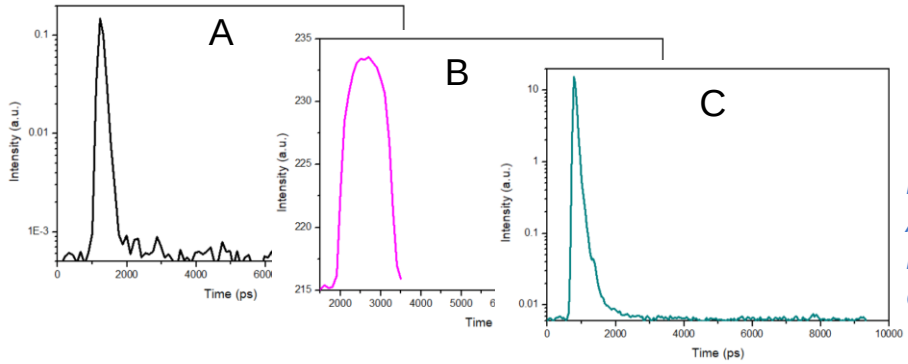
  - **Global binning**: the photons from all the pixels in a region of interest are combined to create a single histogram with more photons

FLIMfit IMPLEMENTATIONS

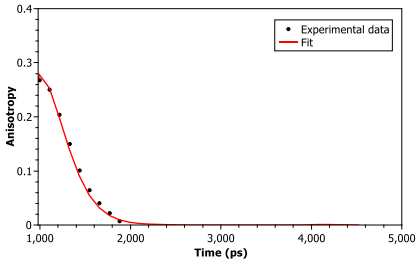
- Loading multidimensional **file formats**:
  - B&H, Picoquant, ome-tiff LaVision Biotec, Leica .pt3, .tiff
  - Single images
  - Series of images: z stacks, time lapses, concentration
  - Multiple channels (time-resolved anisotropy)
- **Instrument response function** (IRF) (image/ascii):
  - **Fluorescent reference**
  - **Scatter** (multiphoton)
- **Background**:
  - **Constant**
  - **Time-varying**
- **Scattered light**
- **Pulse train correction** (i.e. incomplete decays fitting)
- **FRET model** for donors with complex fluorescence decay
- **Time-resolved anisotropy decays analysis**



Loading multidimensional files



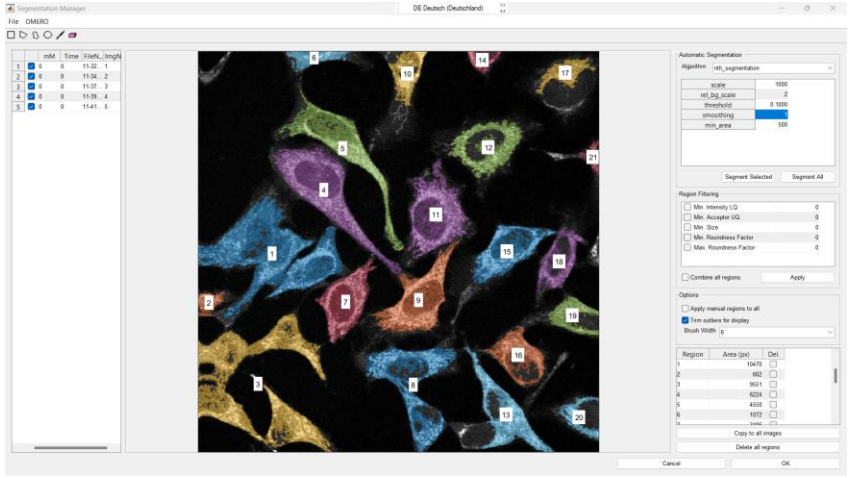
Examples of IRFs of different instruments:
A- scatter (multiphoton TCSPC)
B- erythrosin B in water (time-gated)
C- erythrosin B in water (TCSPC)



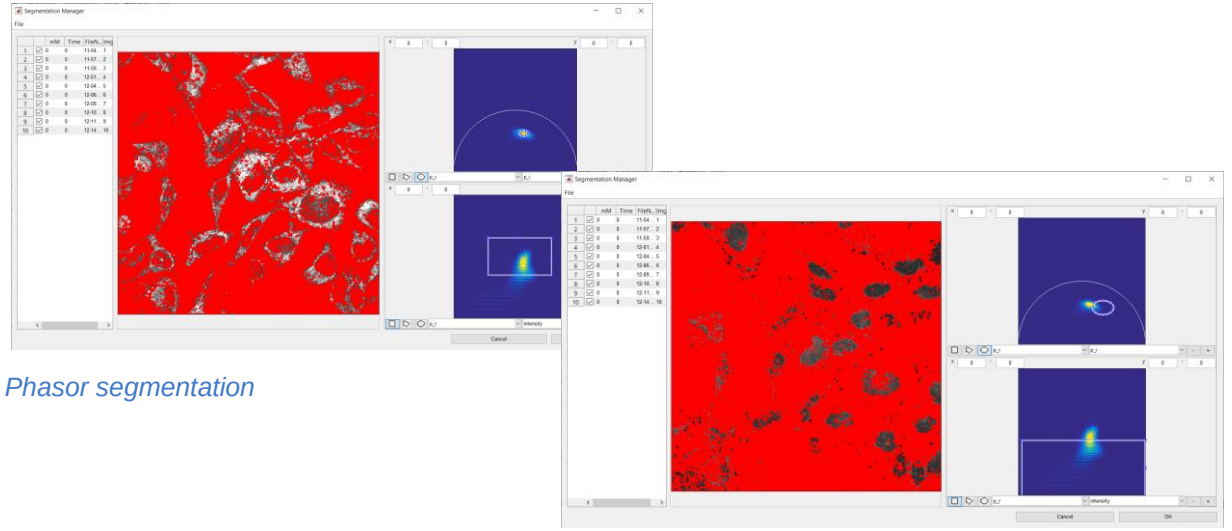
Time resolved anisotropy decay of rhodamine 6G in water

FLIMfit IMPLEMENTATIONS

- **Segmentation**:
  - **Manual**
  - **Automated**: Watershed algorithm + options from Cell Profiler
  - **Acceptor images** (for FRET experiments)
  - **Phasor segmentation** (sine vs. cosine transforms of the decay, or intensity vs. sine/cosine transforms)
  - **Save** and **load** multiple segmentation images using logical operators



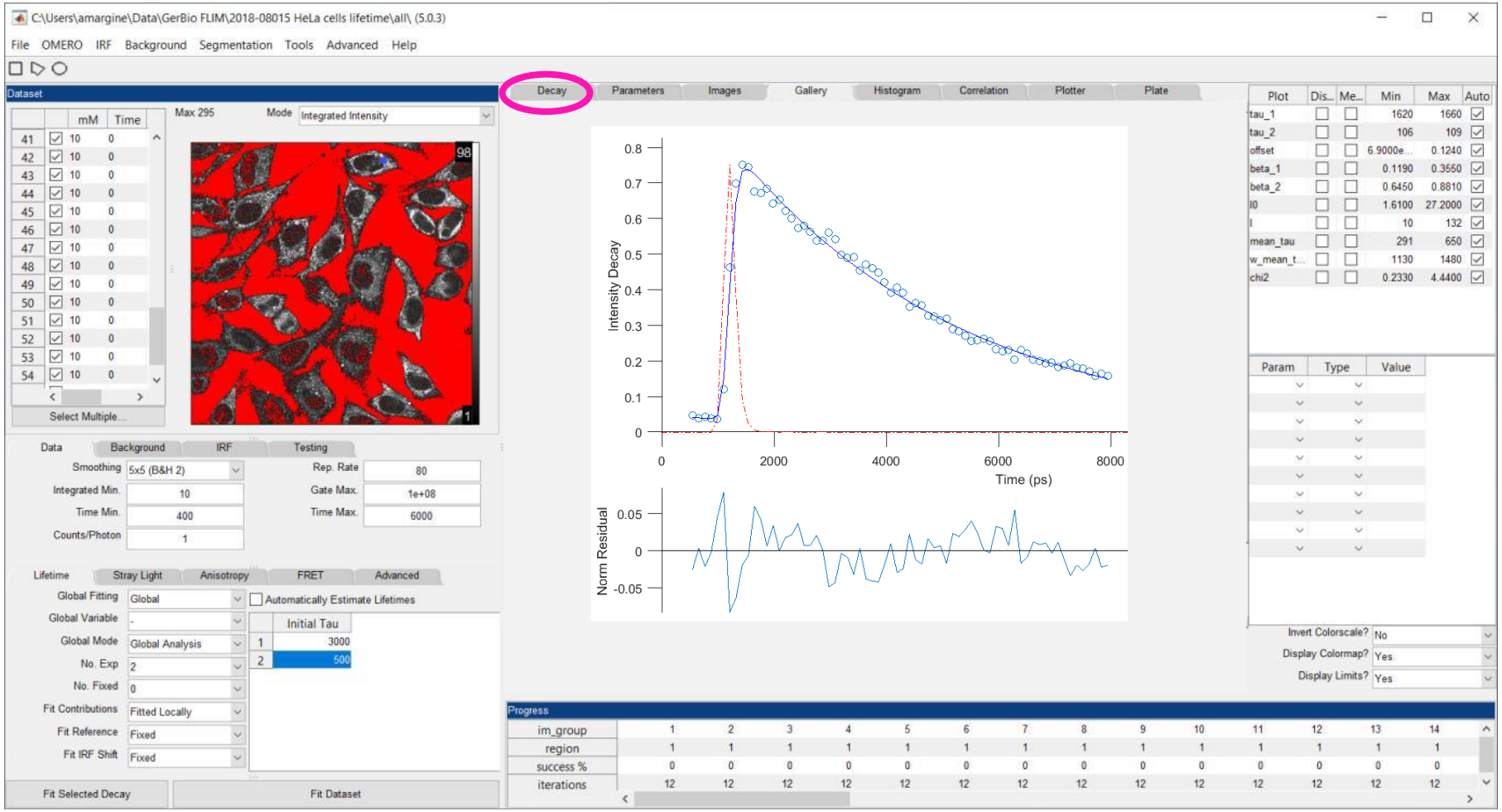
Automated watershed segmentation



Phasor segmentation

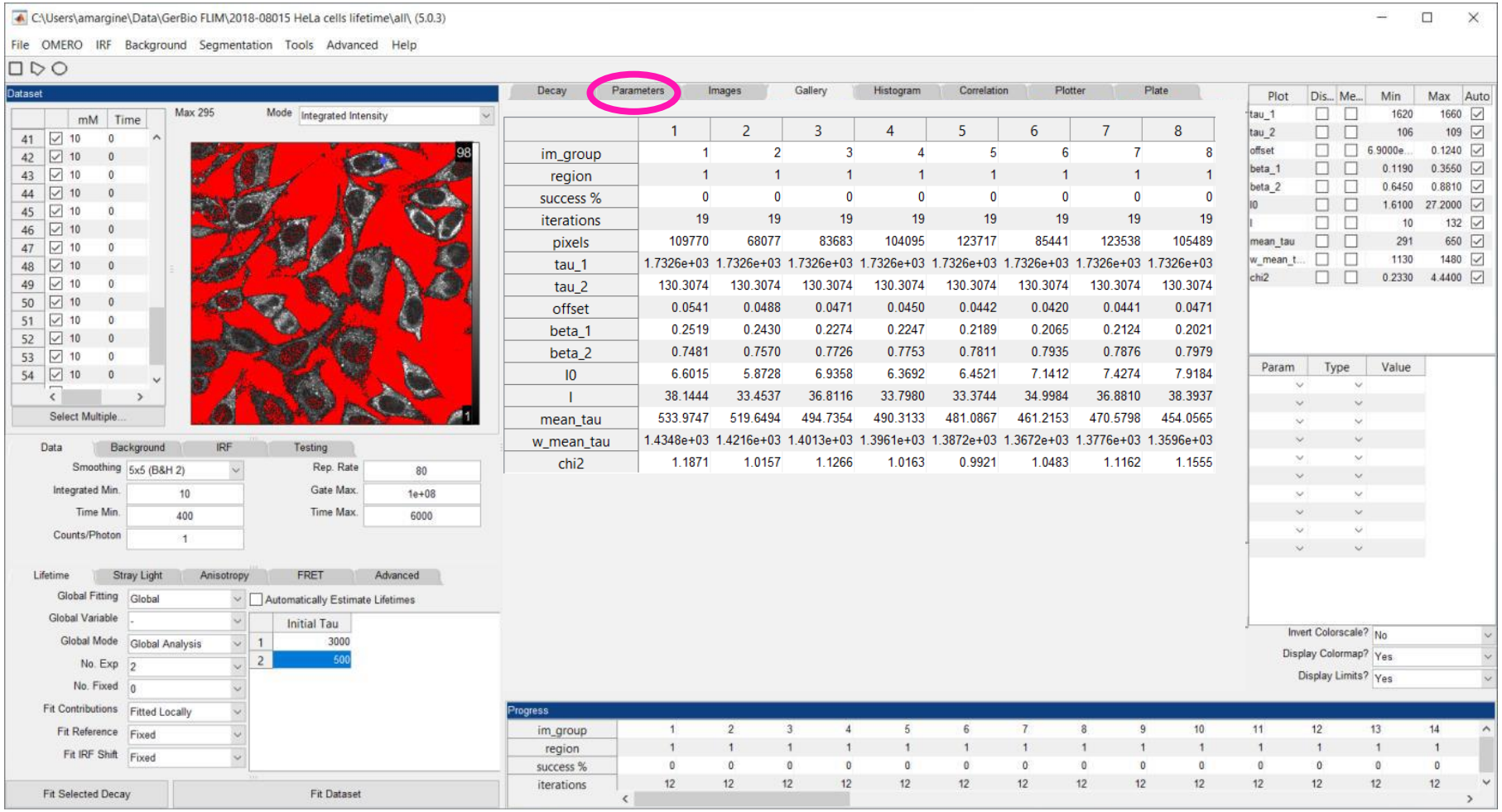
FLIMfit: DATA VISUALISATION

Time resolved fluorescence decay and fit



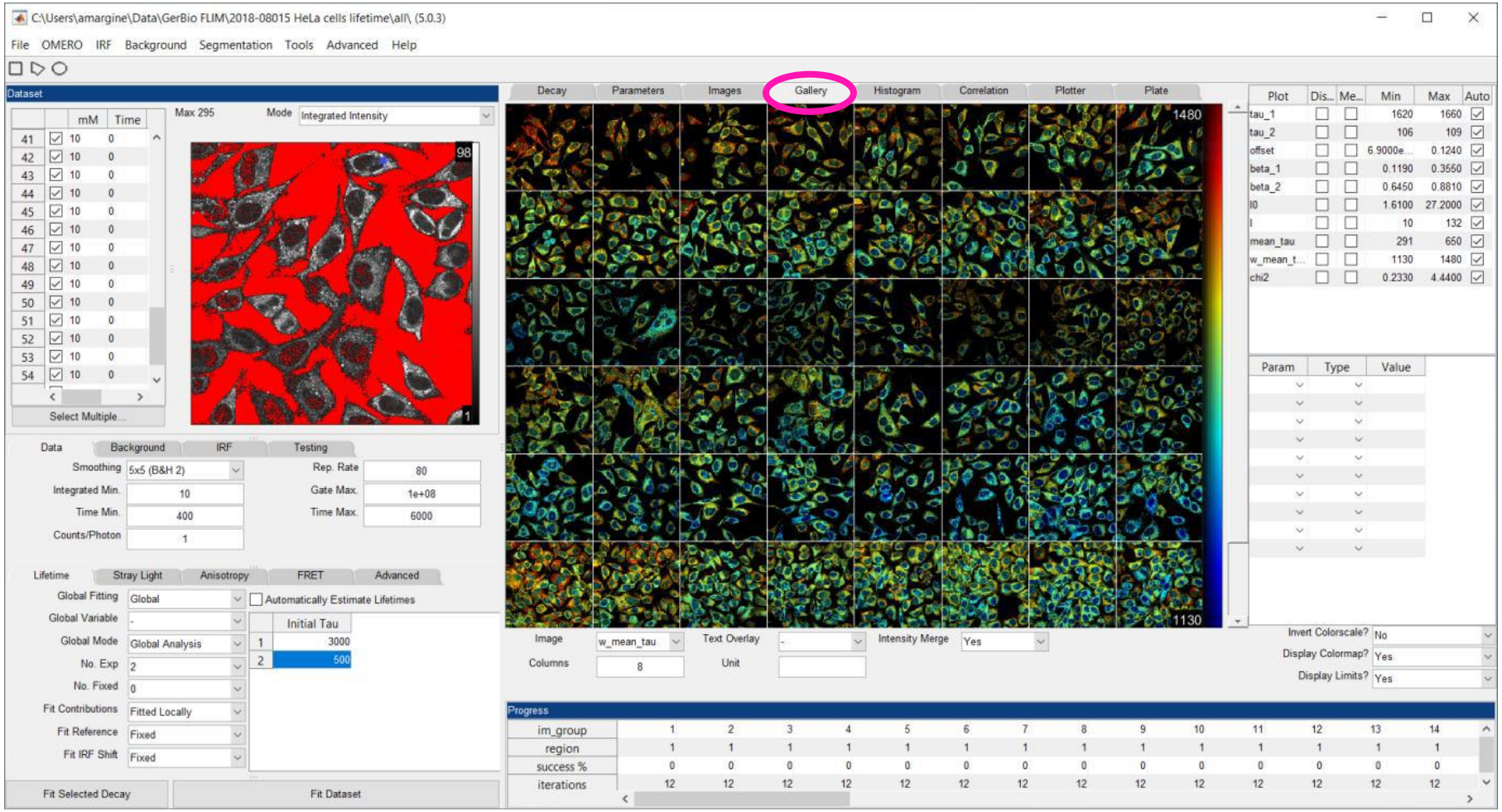
FLIMfit: DATA VISUALISATION

Tabel of fitted parameters (results)



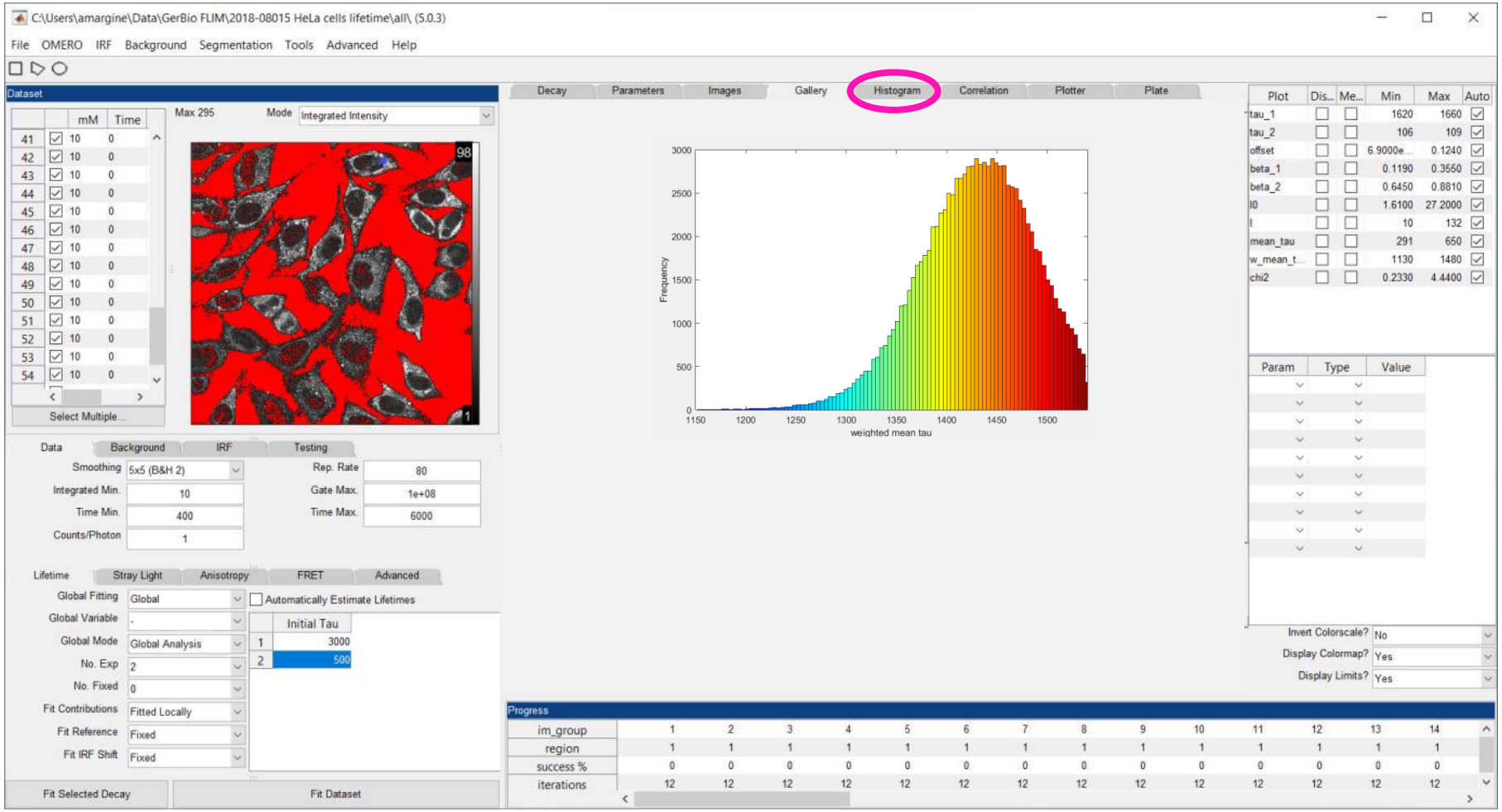
FLIMfit: DATA VISUALISATION

Image gallery of selected fitted parameters



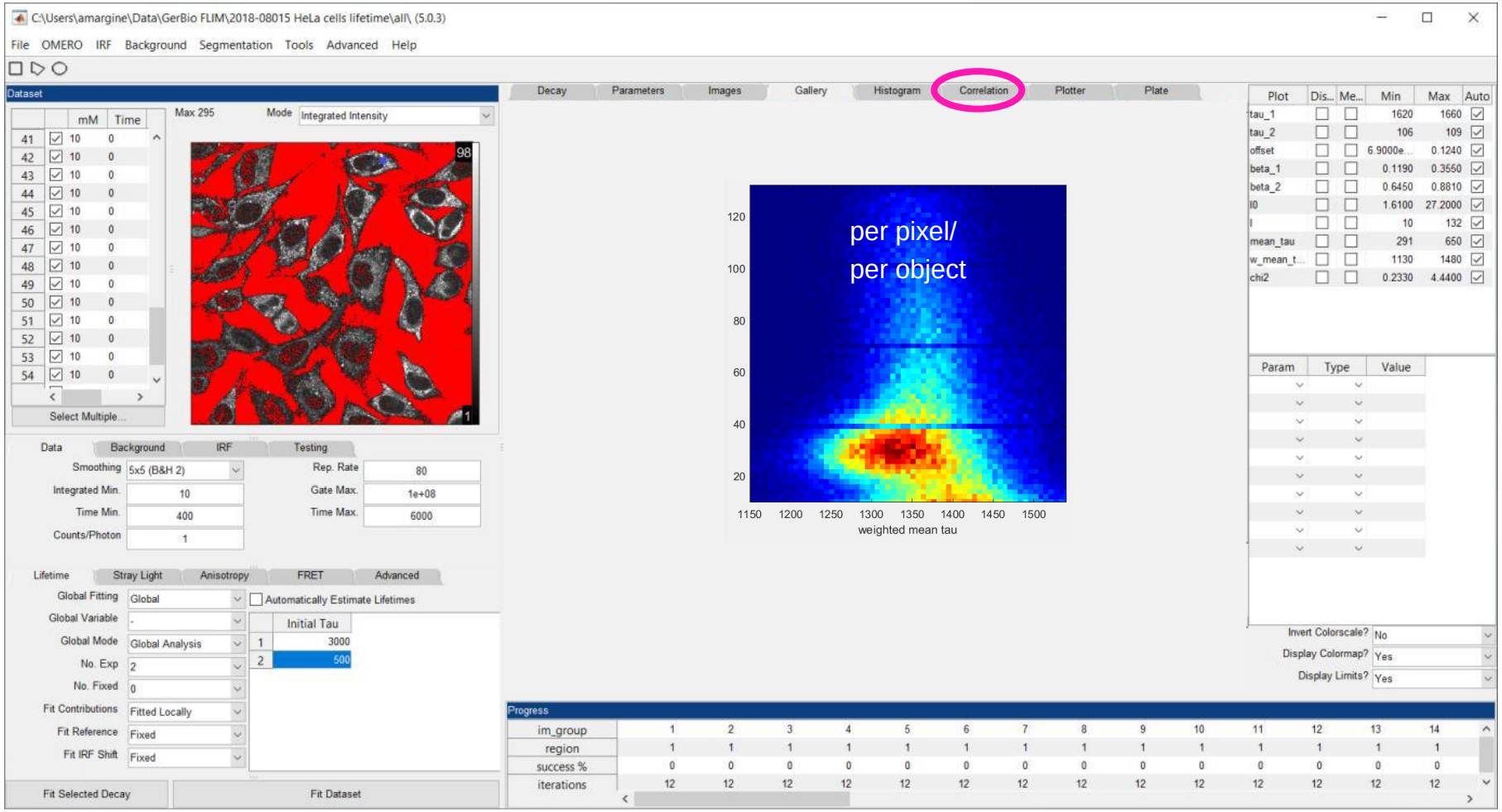
FLIMfit: DATA VISUALISATION

Histograms of selected fitted parameters



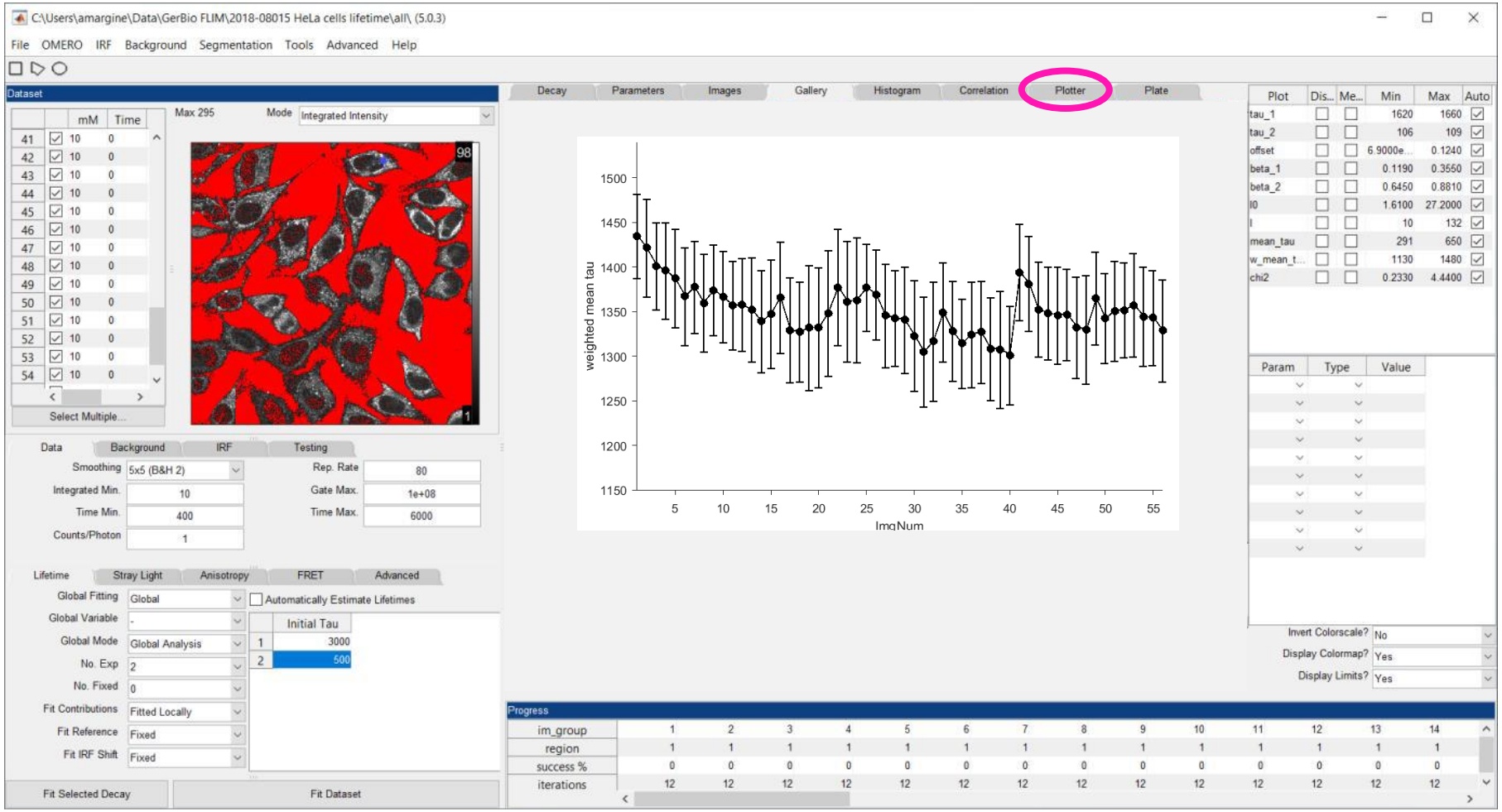
FLIMfit: DATA VISUALISATION

Correlation plots of selected fitted parameters



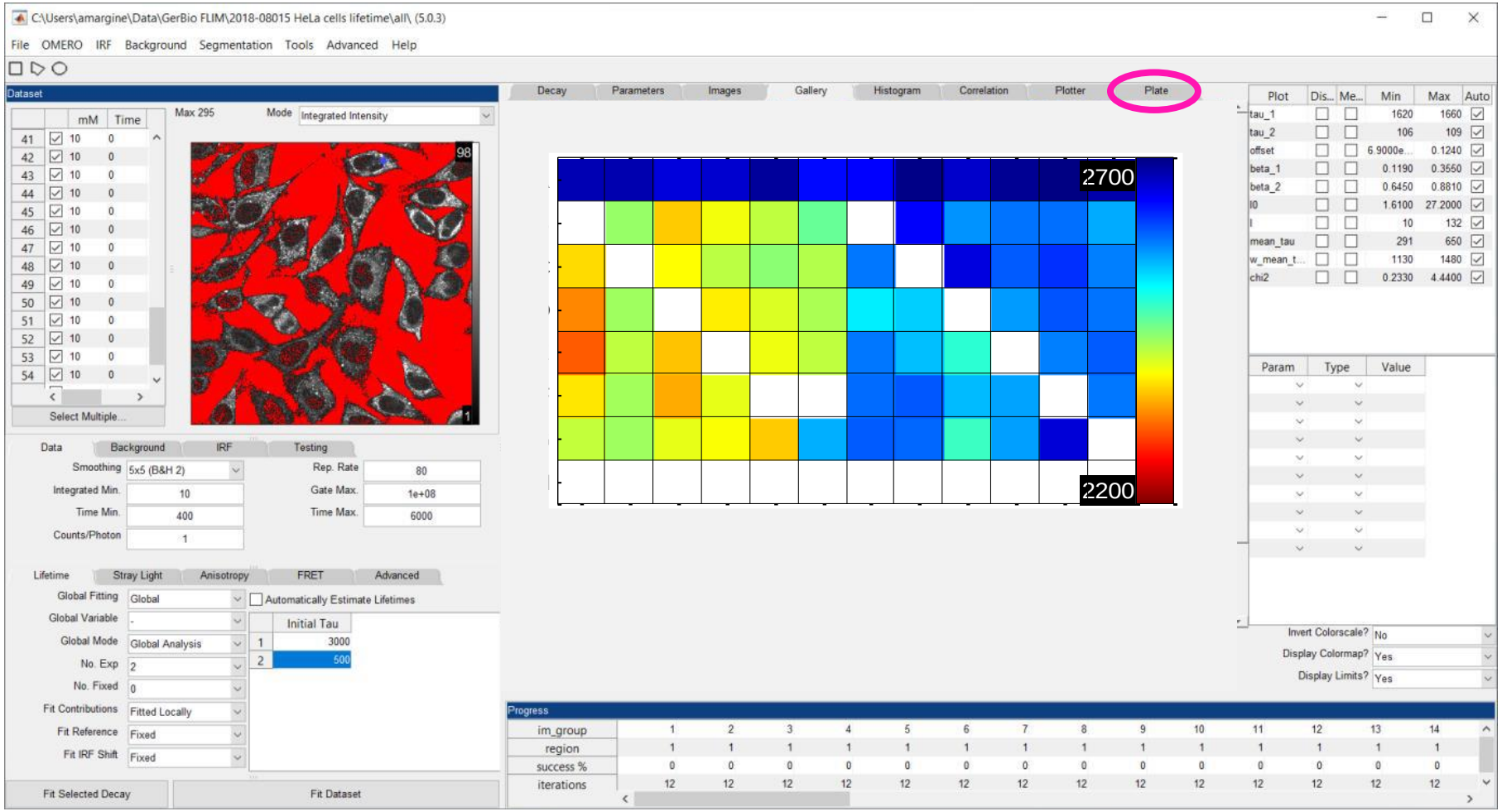
FLIMfit: DATA VISUALISATION

Plots of selected fitted parameters across all images



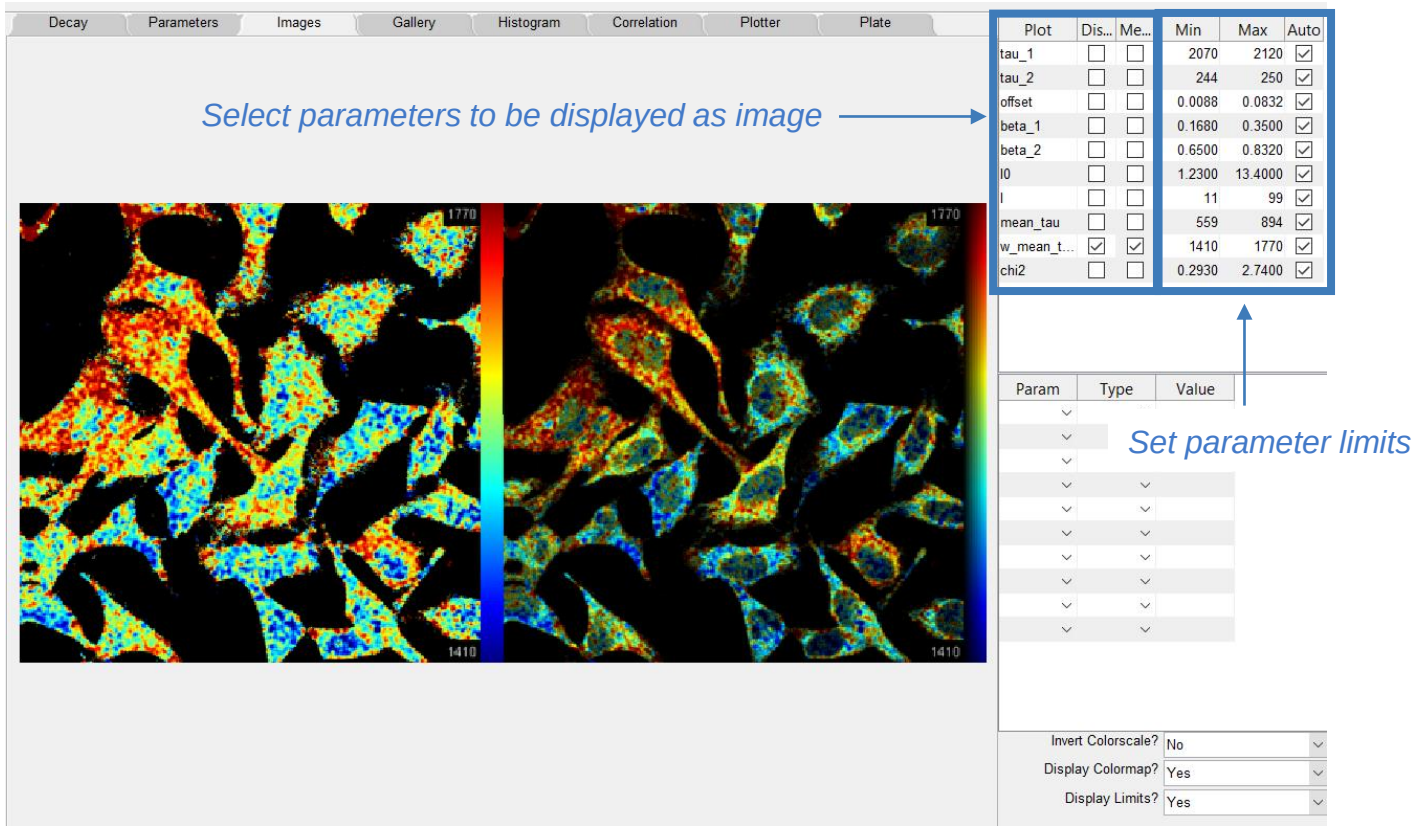
FLIMfit: DATA VISUALISATION

Average values of selected fitted parameters in a 96-well plate



FLIMfit: EXPORTING RESULTS

- Export **images**:
  - FLIM values (.tif, raw 32-bit)
  - FLIM (.tif, RGB)
  - Intensity-weighted FLIM (.tif, RGB)
  - Intensity (.tif, 16-bit)
- Export **results table** (.csv)
- Export **histograms** and **plots** (.csv)
- Export to **Power Point**:
  - Decay + IRF
  - Galerie
  - Histogram
  - Correlation plots
  - Plots



Select and adjust the parameters to be displayed as image

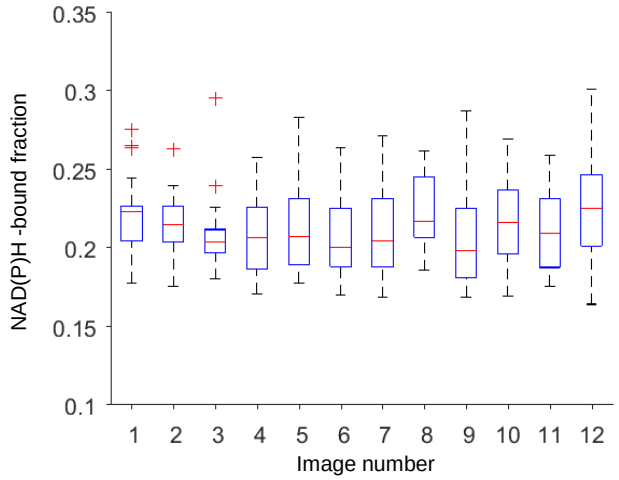
BIOLOGICAL APPLICATIONS OF FLIM GLOBAL ANALYSIS

1. Estimate the free and bound NAD(P)H fractions in live cells

1. Global analysis with double exponential decay

- Set the global analysis for all the images acquired in a given condition (i.e. tissue type, cell type, substrate, inhibitors concentrations etc.)
- The short NAD(P)H lifetime corresponds to the free form
- The long NAD(P)H lifetime corresponds lifetime to the bound form
- Segment the images using the watershed segmentation available in FLIMfit to get statistics per cell

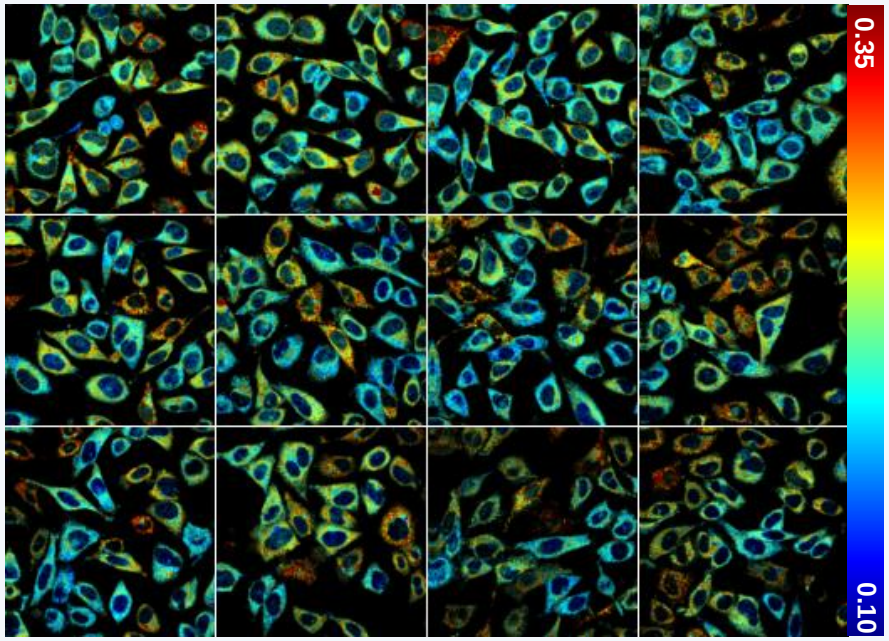
2. Display the results (e.g. images, statistical graphs)



The bound NAD(P)H fraction in HeLa cells metabolising lactate:

- *Box plot of bound fraction/cell/image (left)*
- *Intensity merged images of the bound fraction (right)*

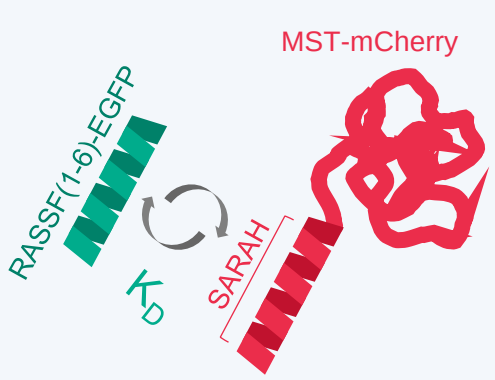
(A. Margineanu, Max Delbrück Centrum Berlin)

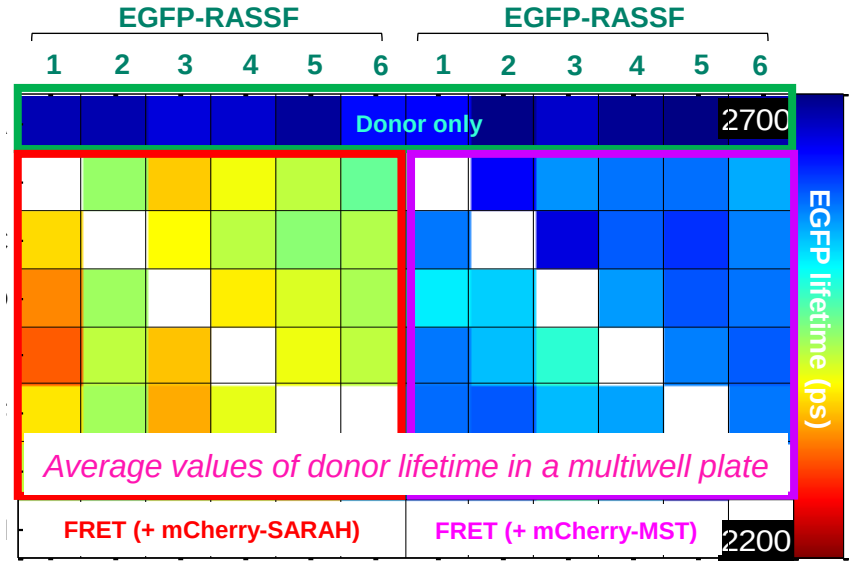


BIOLOGICAL APPLICATIONS OF FLIM GLOBAL ANALYSIS

2. Estimate dissociation constants (K_D) using intermolecular FLIM-FRET

The biological interactions explored by FRET





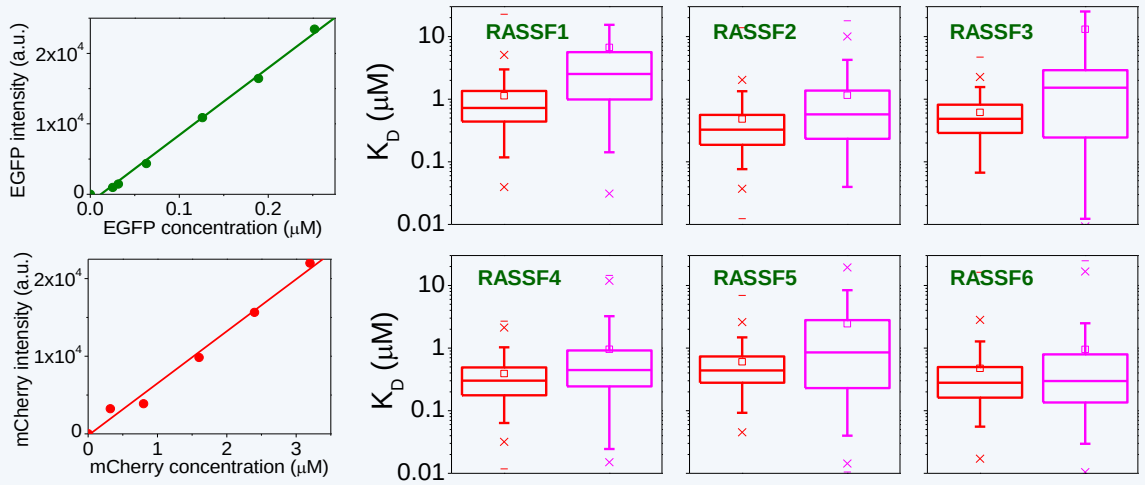
Summary of the K_D calculations

- Find the bound fraction β from the global double exponential fit of the FRET decays

$$I_{(t)} = I_0 \left[(1-\beta)e^{-\frac{t}{\tau_D}} + \beta e^{-\frac{t}{\tau_{DA}}}\right]$$

- Calibrate the EGFP and mCherry intensity vs. concentrations

⇒ Determine the **dissociation constants K_D**: $K_D = \frac{[D_{free}][A_{free}]}{[DA]}$



Estimated values of the intracellular K_D

(statistics per cell):

- RASSF(1-6) with the SARAH domain (red box plots)

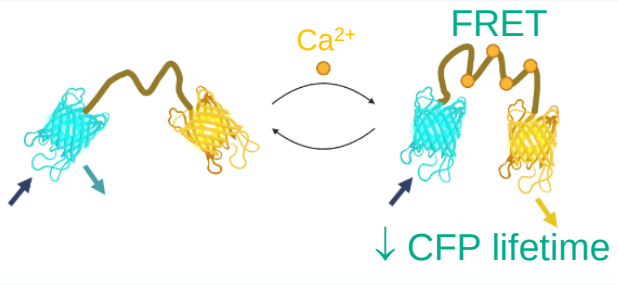
- RASSF(1-6) with the full length MST (magenta box plots)

Margineanu et al. (2016), Sci Rep. **6**:28186, doi: 10.1038/srep28186

BIOLOGICAL APPLICATIONS OF FLIM GLOBAL ANALYSIS

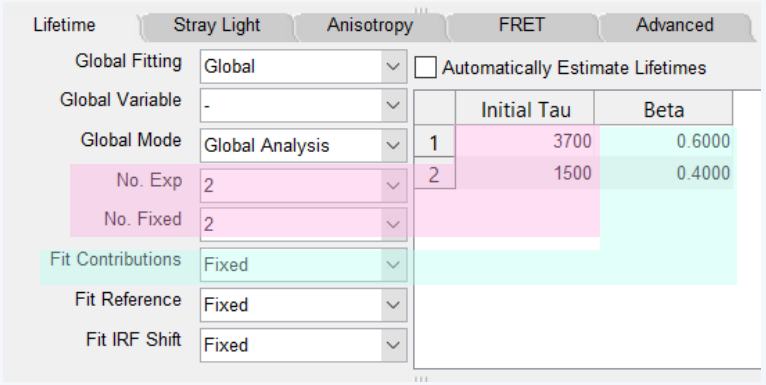
3. Estimate the free and bound fractions of a Ca^{2+} biosensor labeled with a multiexponential donor using the FRET model

CerTn-L15 Ca^{2+} biosensor (Cerulean-Citrine)

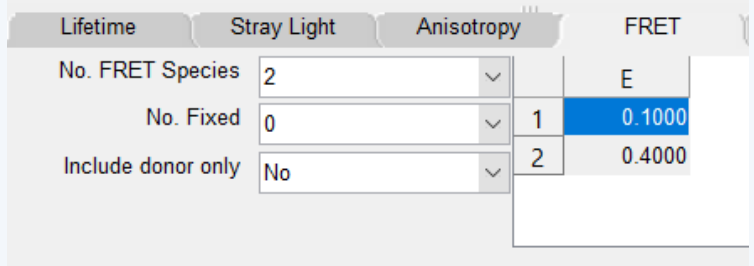


1. Multiexponential donor (Cerulean)

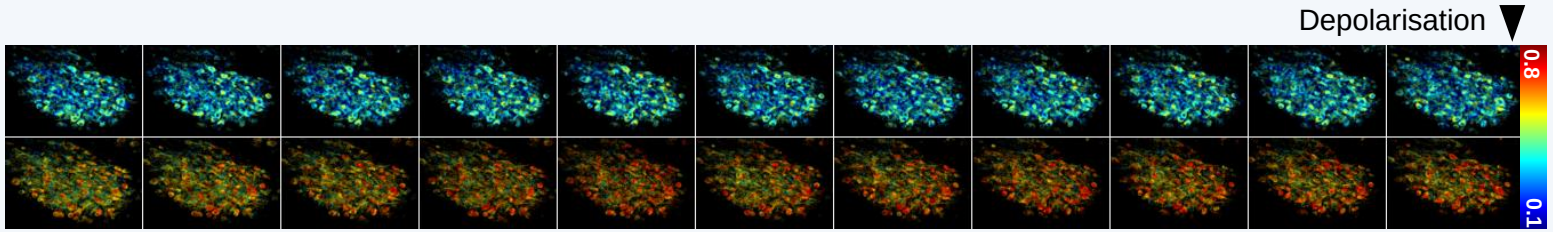
Get the values of the lifetimes and contributions of the donor not interacting with the acceptor and fix them



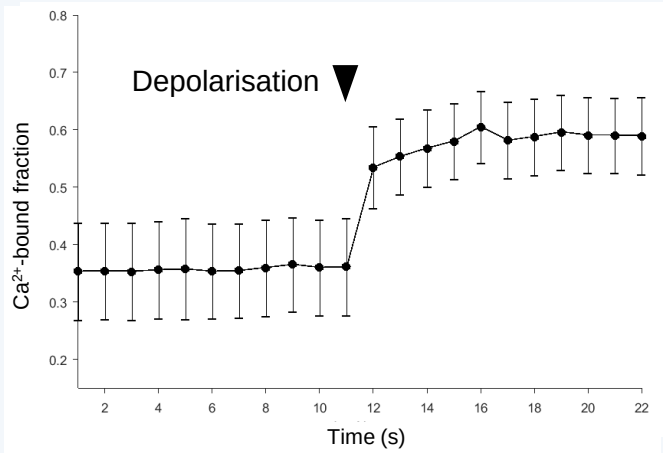
2. Set 2 FRET species with low (Ca^{2+}- free) and high (Ca^{2+}-bound) FRET efficiencies



3. Display and plot the fraction of the component with high FRET efficiency (Ca^{2+}-bound)



Time series of increased Ca^{2+} signalling in live mouse neurons after KCl depolarisation (V. Siffrin, A. Margineanu, Max Delbrück Centrum Berlin)

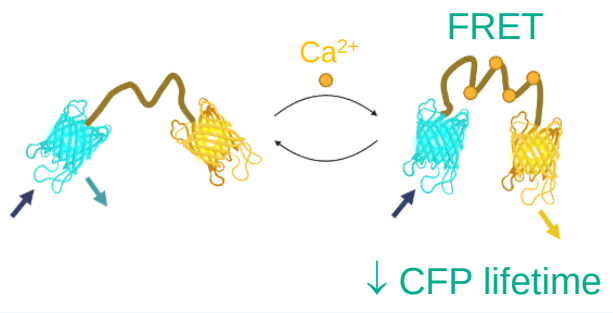


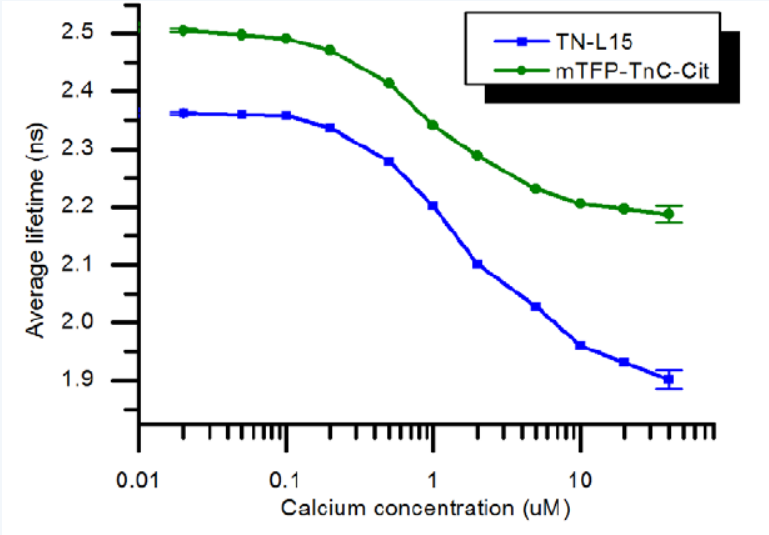
BIOLOGICAL APPLICATIONS OF FLIM GLOBAL ANALYSIS

4. Estimate the intracellular Ca^{2+} concentration from the average lifetime of a FRET biosensor

1. Calibrate the average lifetime of a multiexponential donor vs. Ca^{2+} concentration

Tn-L15 Ca^{2+} biosensor (CFP-YFP)

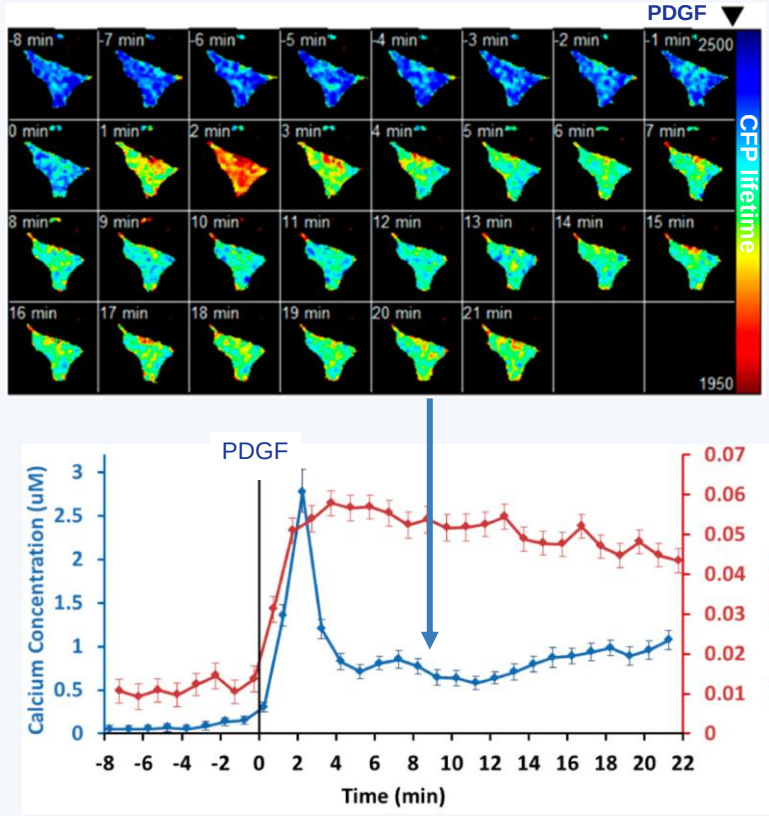




Calibration curve

Laine et al. (2012), PLoS ONE 7(11): e49200. doi:10.1371/journal.pone.0049200

2. Estimate the intracellular Ca^{2+} concentrations



Determine the average CFP lifetime in cells by global analysis

Interpolate on the calibration curve to find the corresponding Ca^{2+} concentrations

Warren et al. (2015), *Int. J. Mol. Sci.*, **16**: 14695

ACKNOWLEDGMENTS

The FLIMfit developers team

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Dr. Yuriy Alexandrov

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