EasyFRAP-web: a web-based tool for the analysis of Fluorescence Recovery After Photo-bleaching (FRAP) data.

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Manual Appendix

APPENDIX – DEFINITIONS

In this appendix, we will provide definitions for the terms used throughout the application. To perform FRAP data processing, the following measurements are necessary (Fig. 1):

- $I(t)^{ROI1}$:the fluorescence intensity in ROI1 (region of interest)
- $I(t)^{ROI2}$:the fluorescence intensity in ROI2 (whole cell)
- $I(t)^{ROI3}$:the fluorescence intensity in ROI3 (background)

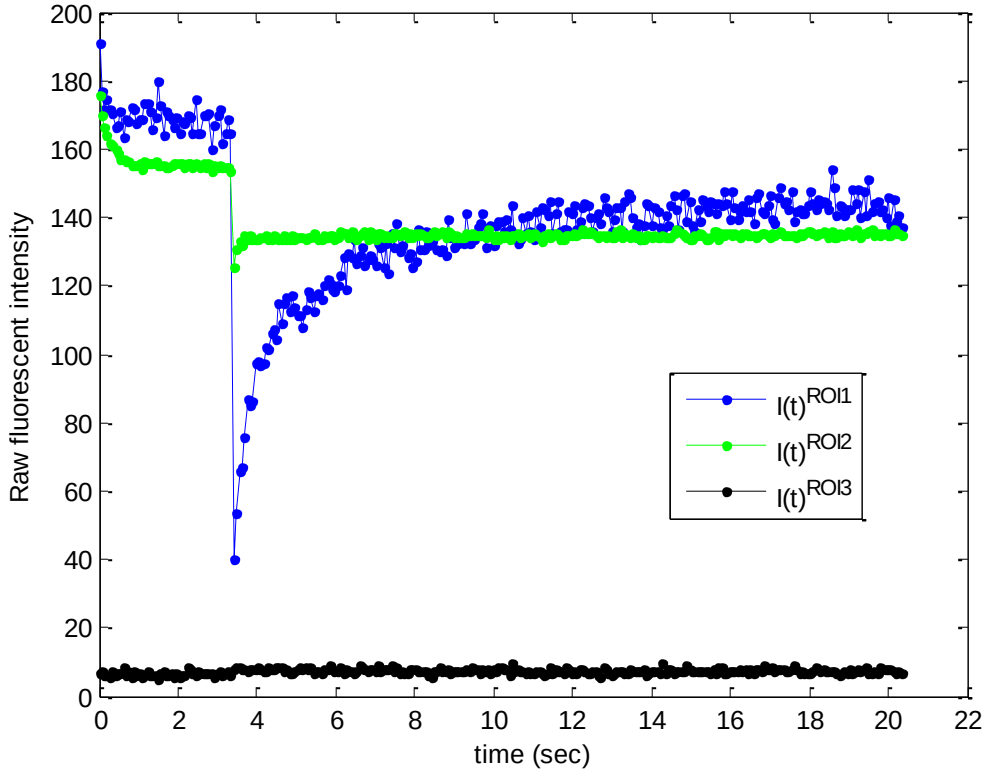

Figure 1: Recovery curves for all ROIs

The first step before data processing is the subtraction of the background intensity from all measurements.

We now have:

- $I(t)_{ROI1'} = I(t)_{ROI1} - I(t)_{ROI3}$
- $I(t)_{ROI2'} = I(t)_{ROI2} - I(t)_{ROI3}$

1. Bleaching depth - Gap Ratio

Bleaching depth is defined as the difference between the mean fluorescence intensity in ROI1 before the bleach and the fluorescence intensity in ROI1 at the first post-bleach time point (time of the bleach), normalized with respect to the first (Fig. 2) and it indicates the efficiency of the bleaching process. A bleaching depth of 1 corresponds to complete bleaching in the region of interest while a bleaching depth of 0 corresponds to no bleaching of molecules in the region of interest.

Bleaching depth is computed using the following formula:

$$bd = \frac{\left(\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{ROI1'} \right) - I(t_{bleach})_{ROI1'}}{\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{ROI1'}}$$

where t_{bleach} the time of the bleach and n_{pre} is the number of pre-bleach images.

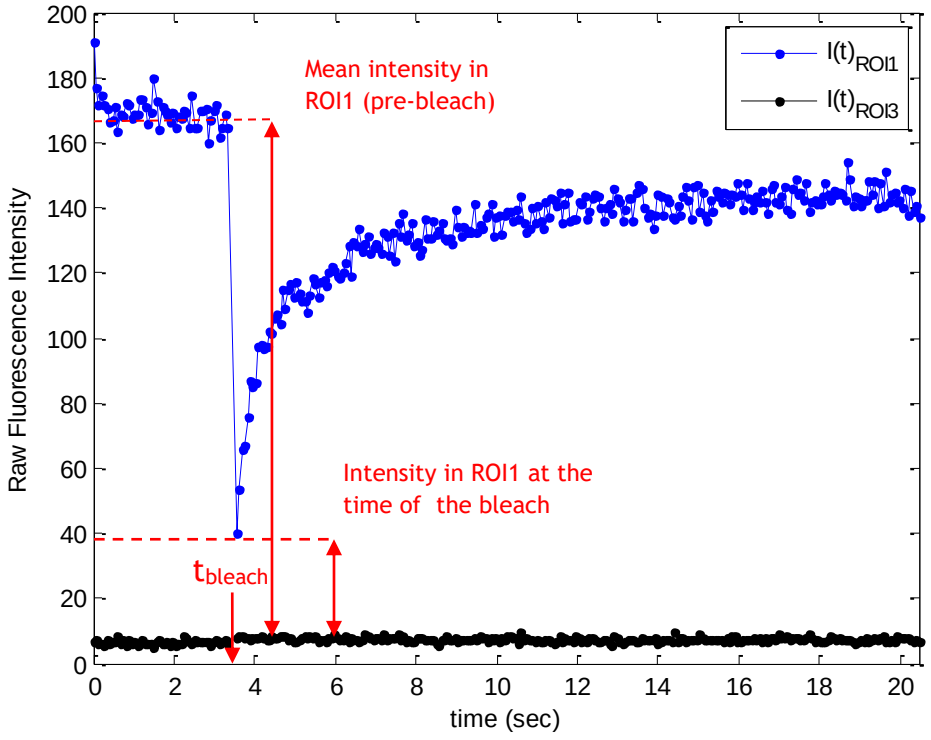

Figure 2: Bleaching depth

Gap ratio is the ratio of the mean fluorescence intensity in ROI2 after the bleach (using 10 after bleaching time points) to the mean fluorescence intensity in ROI2 before the bleach (Fig. 3) and thus it represents the total fluorescence remaining following the bleaching step. A gap ratio of 1 corresponds to no loss of total fluorescence while a gap ratio of 0 corresponds to complete loss of fluorescence. Gap ratio is computed using the following formula:

$$gr = \frac{\frac{1}{10} \cdot \sum_{t_{bleach}}^{t_{bleach+10\Delta t}} I(t)_{ROI\,2'}}{\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{ROI\,2'}}$$

where n_{pre} the number of images before the bleach.

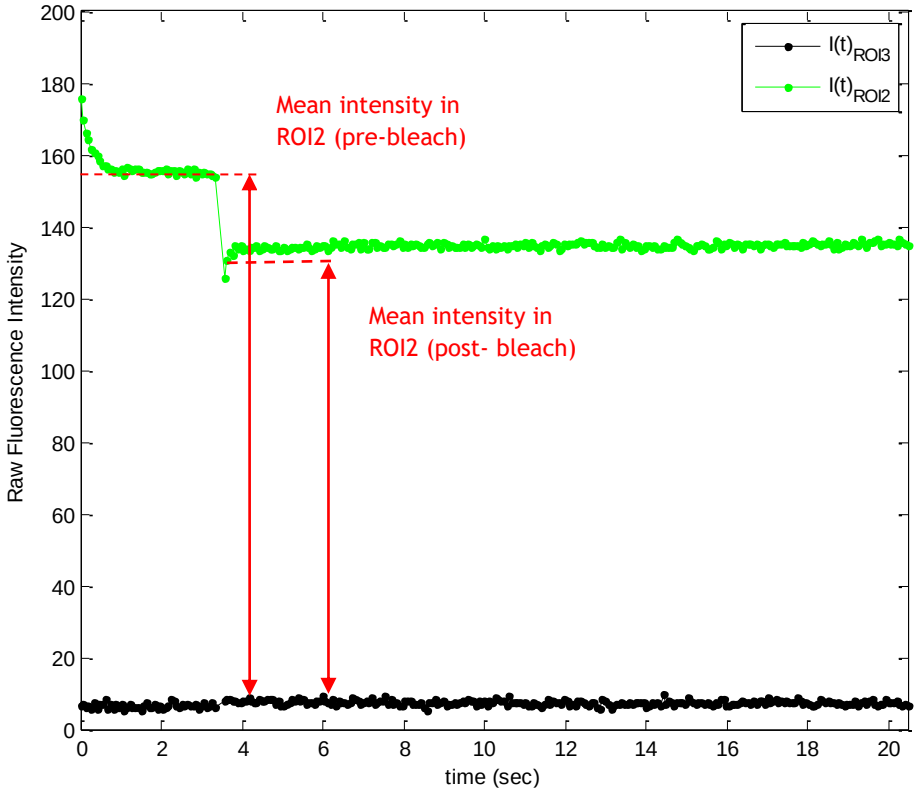

Figure 3: Gap ratio

2. Normalization:

Double normalization corrects for:

i. differences in the starting intensity in ROI1 (by dividing intensities in ROI1 at each time point with the average pre-bleach intensity in ROI1)

ii. differences in total fluorescence during the time course of the experiment due to acquisition bleaching or fluctuations in laser intensity (by dividing with the total fluorescence intensity at each time point (ROI2) and multiplying with the average total pre-bleach intensity)

Double normalization uses the following formula:

$$I(t)_{norm}^{double} = \left(\frac{\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{ROI\,2'}}{I(t)_{ROI\,2'}} \right) \cdot \left(\frac{I(t)_{ROI1'}}{\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{ROI1'}} \right)$$

Full scale normalization additionally corrects for differences in bleaching depth by subtracting the intensity of the first post-bleach image in ROI1.

Full scale normalization is defined according to the following formula:

$$I(t)_{norm}^{fullscale} = \frac{I(t)_{norm}^{double} - I\left(t_{postbleach}\right)_{norm}^{double}}{1 - I\left(t_{postbleach}\right)_{norm}^{double}}$$

where $I\left(t_{postbleach}\right)_{norm}^{double}$ is the first post-bleach value of the double normalized data.

Full scale normalization differs from double normalization in that the recovery curve starts from zero values (Figure 4 and 5). With both types of normalization, curves will reach 1 if there is no immobile fraction (Figure 4) and values less than 1 when an immobile fraction is present (Figure 5).

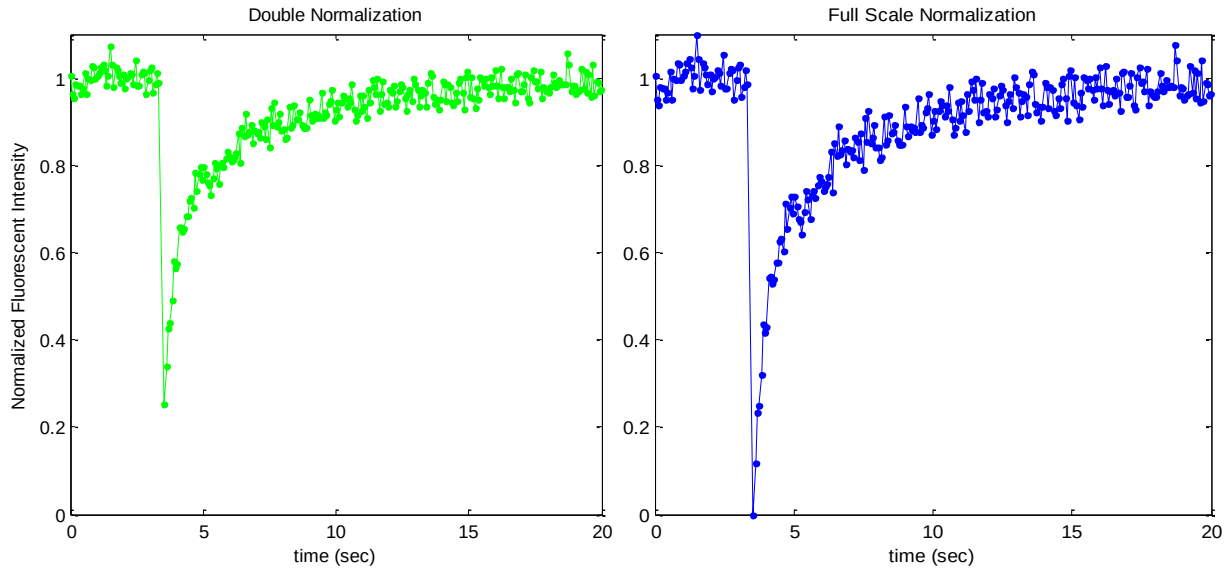

Figure 4: Different Normalization methods with no immobile fraction

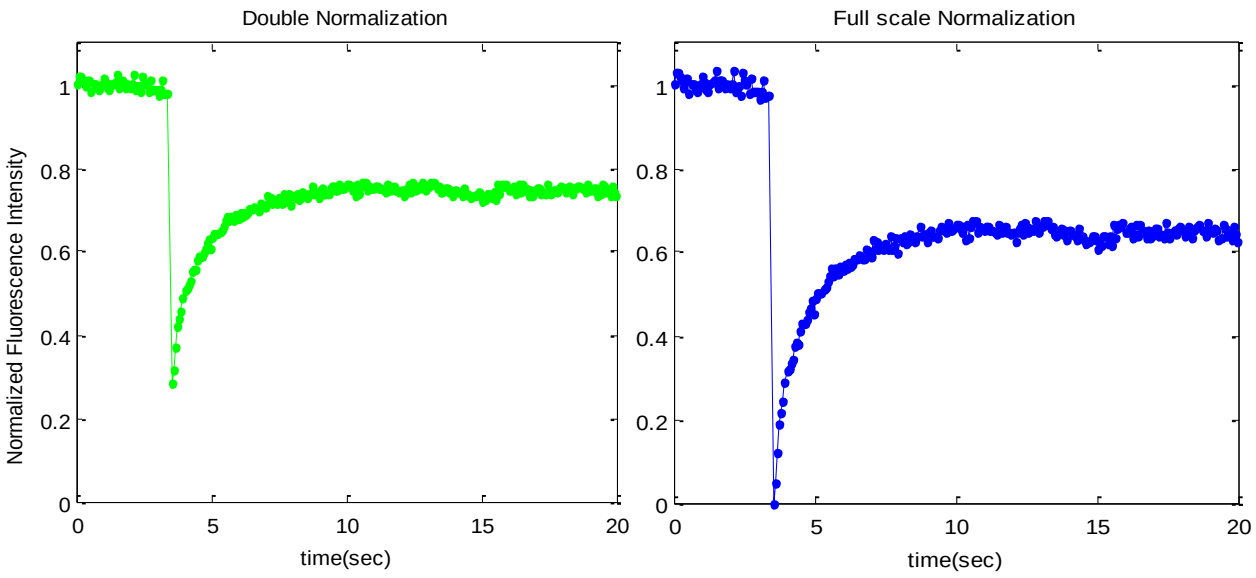

Figure 5: Different Normalization methods with immobile fraction

3. Fitting process:

The single and double term equations used to fit the data are:

- $I_{fit1} = I_0 - a \cdot e^{-\beta \cdot t}$
- $I_{fit2} = I_0 - a \cdot e^{-\beta \cdot t} - \gamma \cdot e^{-\delta \cdot t}$

For the curve fitting we execute R code through SQL Server. More specifically, we use R's non-linear least squares (nls) function. The optimization algorithm used in the fitting procedure is the NL2SOL algorithm. The algorithm parameters are fixed in the following way: the maximum value of fitting iterations is set to 1000, while the starting values of the parameters has been set to $I_0 = 0.85$, $\alpha = 0.5$, $\beta = 0.563$, $\gamma = 0.316$, $\delta = 0.36$. The computation of the parameters of the best-fit curve allows us to estimate the mobile fraction and the t_{half} values.

i. Mobile fraction estimation:

The mobile fraction represents the fraction of molecules that are mobile and free to diffuse whereas the immobile fraction the fraction of molecules that are bound and do not diffuse back into ROI1 (Figure 6). It is estimated using the following formula:

$$mf = \frac{I_\infty - I_a}{1 - I_a}$$

where I_∞ the normalized fluorescence intensity after full recovery and I_α the normalized intensity of the first post-bleach time point (Figure 6). I_∞ is the plateau of the curve and is equal to I_0 for both exponential equations. For full scale normalized curves I_α=0 and from the above formula for mobile fraction this gives:

$$mf = I_0$$

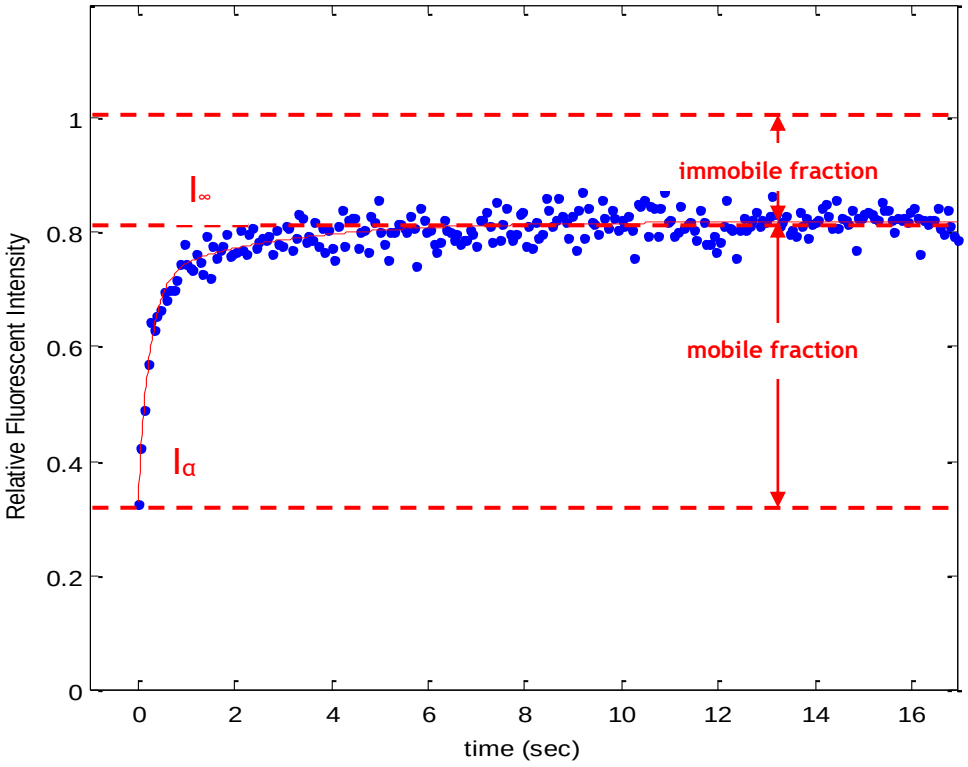

Figure 6: Mobile and immobile fraction

For double normalized curves, the mobile fraction is calculated as follows:

- **Using a single exponential equation:**

For t=0 it is $I_\alpha = I_0 - \alpha$. From the above formula for mobile fraction:

$$mf = \frac{I_\infty - I_a}{1 - I_a} = \frac{I_0 - (I_0 - a)}{1 - (I_0 - a)} = \frac{a}{1 - (I_0 - a)}$$

- **Using a double exponential equation:**

For t=0 it is $I_\alpha = I_0 - \alpha - \gamma$, so:

$$mf = \frac{I_\infty - I_a}{1 - I_a} = \frac{I_0 - (I_0 - a - \gamma)}{1 - (I_0 - a - \gamma)} = \frac{a + \gamma}{1 - (I_0 - a - \gamma)}$$

ii. T_{half} estimation:

The t_{half} value is defined as the half maximal recovery time, i.e. the time when the intensity equals half of the maximal intensity ($I_{fit} = I_0/2$) (Figure 7).

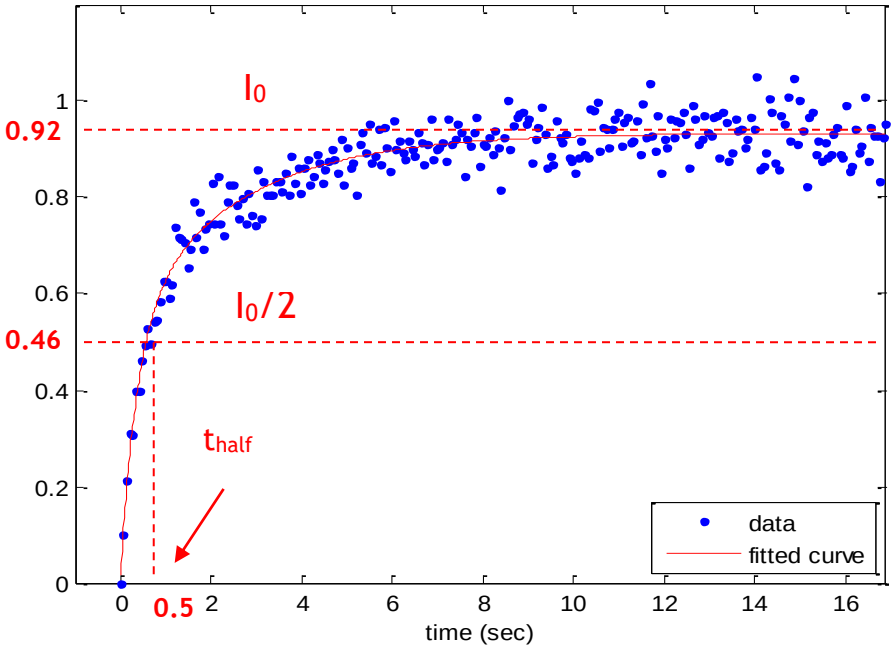

Figure 7: t_{half} for full scale normalized data

For the case of the single exponential term, t_{half} is computed in the following way:

- **Full scale normalization:** For full scale normalized curves, for t=0 it is I_{fit} =0, which means that:

$$I_0 - a \cdot e^{-\beta \cdot 0} = 0 \Rightarrow a = I_0$$

If we solve the exponential equation for t_{half} we get:

$$\frac{I_0}{2} = I_0 - a \cdot e^{-\beta \cdot t_{half}} \Rightarrow e^{-\beta \cdot t_{half}} = \frac{I_0}{2a} \Rightarrow t_{half} = -\ln\left(\frac{I_0}{2a}\right) \Big/ \beta$$

And since $a=I_0$ that gives us:

$$t_{half} = -\frac{\ln\left(\frac{I_0}{2a}\right)}{\beta} = -\frac{\ln\left(\frac{1}{2}\right)}{\beta} = \frac{\ln(2)}{\beta}$$

- **Double normalization:** For double normalized data, the recovery curve does not start from zero intensity and t_{half} is defined as the time when $I_{fit}=(I_0+I_a)/2$, where I_a the intensity of the first recovery point (Figure 8).

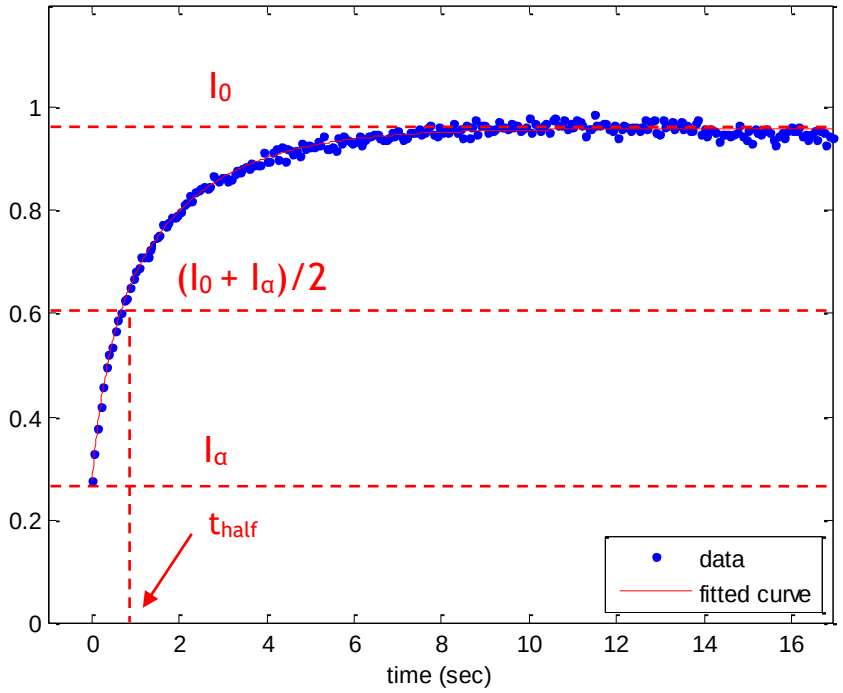

Figure 8: t_{half} for double normalized data

In this case, t_{half} is computed as follows:

$$\frac{I_0 + I_a}{2} = I_0 - a \cdot e^{-\beta \cdot t_{half}} \Rightarrow \frac{2I_0 - a}{2} = I_0 - a \cdot e^{-\beta \cdot t_{half}} \Rightarrow$$

$$\Rightarrow -\frac{a}{2} = -a \cdot e^{-\beta \cdot t_{half}} \Rightarrow e^{-\beta \cdot t_{half}} = \frac{1}{2} \Rightarrow -\beta \cdot t_{half} = \ln(\frac{1}{2}) \Rightarrow$$

$$\Rightarrow t_{half} = \frac{\ln(2)}{\beta}$$

In the case of a double term exponential fitting equation there is no closed form solution and the t_{half} value is computed numerically.

4. Goodness-of-Fit statistics

To compute the R-square we use the following statistical measures:

$$SSE = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2$$

$$SSR = \sum_{i=1}^{n} (\hat{y}_i - \bar{y})^2$$

$$SST = \sum_{i=1}^{n} (y_i - \bar{y})^2$$

where SSR is the sum of squares of the regression, SST the total sum of squares and SSE the sum of squares due to error. Essentially, SSE describes the total deviation of the fitted points from the experimental points and the closer it is to zero, the better the fit. SSR and SST are the sum of squares of the regression and the total sum of squares and it is obvious that:

$$SSE = SSR + SST$$

So R-square is defined as follows:

$$R - square = \frac{SSR}{SST} = 1 - \frac{SSE}{SST}$$

R-square varies from 0 to 1, with a value closer to 1 indicating that the fit explains better the total variation in the data. If R-square equals 1, it means that SSE=0 i.e. the approximated points fit perfectly the experimental points. On the other hand, if R-square equals 0, it means that SSE=SST, i.e. the approximated points are no different than the mean, so the approximation is random.