

# Supplementary Methods - mathematical derivations

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## 1 Model definition

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The fluorophore maturation kinetics was described as a one-step process. Each fluorescence channel was modelled separately but due to tandem timer design each channel shared the same constant protein production rate  $p$  and constant degradation rate  $k$ . The time-dependent rate equations used are therefore

$$\begin{aligned} \dot{X}_i^0(t) &= p - (k + m_i)X_i^0(t) \\ \dot{X}_i(t) &= m_i X_i^0(t) - kX_i(t) \end{aligned} \tag{1}$$

where  $X_i^0(t)$  and  $X_i(t)$  are the molecular populations of the non-mature and mature fluorophore populations respectively at time  $t$  for the  $i$ th fluorescence channel with  $i \in \{1, 2\}$ . We chose the convention that  $i = 1$  is the fast-maturing fluorescence channel and  $i = 2$  is the slow-maturing fluorescence channel.

## 2 Model solutions

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The time-dependent solutions to eq. 1 with the boundary conditions  $X_i^0(0) = 0$  and  $X_i(0) = 0$  were calculated to be

$$\begin{aligned} X_i^0(t) &= p(1 - e^{-(k+m_i)t})/(k + m_i) \\ X_i(t) &= pe^{-(k+m_i)t}(k - e^{m_it}(k + m_i - e^{kt}m_i))/(k(k + m_i)) . \end{aligned} \tag{2}$$

The corresponding steady state solutions to eq. 2 are

$$\begin{aligned}\lim_{t \rightarrow \infty} X_i^0(t) &= p/(k + m_i) \\ \lim_{t \rightarrow \infty} X_i(t) &= pm_i/(k(k + m_i)) .\end{aligned}\tag{3}$$

### 3 Timer ratio with FRET

Fluorescence intensity  $I_i$  is proportional to the number of mature fluorescent molecules  $X_i$ . If FRET occurs between channel 1 and channel 2 the fluorescence intensity of channel 1 will be reduced by an amount proportional to the FRET efficiency  $E$  and the proportion  $b$  of channel 2 fluorophores available as acceptors. In the time-dependent model this was described as

$$\begin{aligned}I_1(t) &= f_1 X_1(t)(1 - b(t)E) \\ I_2(t) &= f_2 X_2(t)\end{aligned}\tag{4}$$

where the proportionality constant  $f_i$  incorporates multiplicative effects such as fluorophore brightness and quantum yield, and  $b(t) = X_2(t)/(X_1^0(t) + X_2(t))$ . We did not consider FRET from channel 2 to channel 1 since it is physiologically improbable to encounter such cases as slower-maturing fluorophores tend to have longer wavelengths than faster-maturing fluorophores.

The time-dependent timer ratio  $R$  incorporating FRET was defined as

$$R(t) = I_2(t)/I_1(t) = fX_2(t)/(X_1(t)(1 - b(t)E))\tag{5}$$

where  $f = f_2/f_1$ .

In full the the timer ratio is therefore

$$R(t) = f \frac{e^{(m_1 - m_2)t}(k + m_1)(k - e^{m_2 t}(k + m_2 - e^{kt}m_2))}{(k - e^{m_1 t}(k + m_1 - e^{kt}m_1))(k + m_2)(1 - E \frac{k - e^{-m_2 t}k + m_2 - e^{kt}m_2}{(1 - e^{kt})(k + m_2)})}\tag{6}$$

, which in steady state reduces to

$$\lim_{t \rightarrow \infty} R(t) = f \frac{m_2(k + m_1)}{m_1(k + m_2 - Em_2)} .\tag{7}$$

### 4 Time to reach steady state

The time to reach steady state for the ratio was determined from the kinetics of the slower maturing fluorophore, FP2. Since FP2 is not affected by FRET from FP1 we may calculate this either from the fluorescence intensity in eq. 4 or the molecular population in eq. 2. Here we focus on the latter. We choose to define the time to reach steady-state as the point of intersection chose the line tangent to the point of inflection and the steady state value (see Fig. S4A) given by eq. 3. The time coordinate  $t^*$  at the point of inflection was calculated to be

$$t^* = (1/m_2) \log(1 + m_2/k) .\tag{8}$$

The slope  $s$  of the tangent to the FP2 profile is given by

$$s = pm_2(1 + m_2/k)^{k/m_2}/(k + m_2) .\tag{9}$$

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Calculating the point of intersection between the tangent line and the steady state value led to the following definition for the time to reach steady state,  $T_{ss}$ .

$$T_{ss} = 1/k + 1/(k + m_2) + \log(1 + m_2/k)/m_2 \quad 10$$

## 5 Timer signal

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The timer signal was defined between two protein half-lives,  $T_{1/2}^A$  and  $T_{1/2}^B$  where  $T_{1/2}^B > T_{1/2}^A$ . We considered the log fold-change between the corresponding timer ratios  $R^B = I_2^B/I_1^B$  and  $R^A = I_2^A/I_1^A$ .

To investigate the effect of background noise on our ability to detect differences in timer signal we defined the following additive error model.

$$S = \log_2 \left( \frac{I_2^B + \epsilon_2^B}{I_1^B + \epsilon_1^B} \Big/ \frac{I_2^A + \epsilon_2^A}{I_1^A + \epsilon_1^A} \right) \quad 11$$

where  $\epsilon_1^A, \epsilon_2^A, \epsilon_1^B, \epsilon_2^B \sim N(0, \sigma^2)$  are independent. Computer simulations were used to obtain an estimate of the population mean  $\mu_S$  and standard deviation  $\sigma_S$ . From this we formed the coefficient of variation term

$$CV = \sigma_S/\mu_S . \quad 12$$

## 6 Timer signal and FRET

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To explain why an increase in FRET increases timer signal we considered timer signal without additive noise and denoted timer signal without FRET ( $E = 0$ ) as  $D_0$  and timer signal with positive FRET ( $E > 0$ ) as  $D_E$ . We calculated that

$$D_E/D_0 = 1 + \frac{1}{D_0} \log_2 \left( \frac{1 - b^A E}{1 - b^B E} \right) \quad 13$$

where  $b^A$  and  $b^B$  are the proportions of FP2 fluorophores available as acceptors for the shorter-living and longer-living proteins, respectively. Since the population of mature FP2 fluorophores is relatively more abundant for the long-living protein,  $b^B > b^A$ , which implies that  $1 - b^B E < 1 - b^A E$ . Therefore  $\log_2((1 - b^A E)/(1 - b^B E))$  is a positive quantity and eq. 13 is greater than one.