Appendix I

Theory and measurement of GFP photobleaching kinetics

GFP exists in a pH-dependent equilibrium between a dark (D) and fluorescent state (F). Only the fluorescent species is excited by the blue excitation light typically used to image GFP in fluorescence microscopy [1] hence the GFP can only be photobleached when it is present in this form. This can be represented as:

(AI1)

where B represents the bleached species, k_1 and k_{-1} represent the forward and reverse rate constants for formation of the fluorescent form and the ratio k_1/k_{-1} is equivalent to Ka. The rate constant k_b refers to the photobleaching process and will be dependent on the intensity of illumination. The spontaneous interconversion between the dark and fluorescent forms of GFP is known to be reversible and to occur in the submillisecond time regime [1, 2]. If photobleaching occurs on a much slower time scale, the fluorescence intensity *F* will decay monoexponentially:

$$F(t) = F_o \, exp\left(-t / \tau_{obs}\right) \qquad \text{(AI2)}$$

where F_o corresponds to the initial intensity and represents the initial pH dependency of the fluorescence intensity as described by Eq (1). The parameter τ_{obs} represents the reciprocal of the effective or observed rate constant for photobleaching (k_{obs}). This parameter is directly proportional to the proton concentration:

$$\tau_{obs} = \frac{1}{k_{obs}} = [H^+]\left(\frac{\tau_b}{K_a}\right) + \tau_b \qquad \text{(AI3)}$$

where τ_b is the reciprocal of k_b.

1 Kneen, M., Farinas, J., Li, Y. and Verkman, A. S. (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. Biophys. J. **74**, 1591-1599

2 Haupts, U., Maiti, S., Schwille, P. and Webb, W. W. (1998) Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy. Proc. Natl. Acad. Sci. USA **95**, 13573-13578

Appendix II

Determination of pH of the digestive vacuole (DV) in a system where a significant population of the GFP chimera is present in other compartments

The general solution describing the pH dependence of GFP fluorescence is given by Eq(1).

The observed fluorescence signal from a native sample (F_n) has fluorescence contributions from the DV (F_{DV}) and vesicular (F_{ves}) populations and a non-fluorescent, pH-independent contribution due to background (B):

$$F_n = F_{DV} + F_{ves} + B = \frac{G_{DV}}{10^{pKa-pH_{DV}}+1} + \frac{G_{ves}}{10^{pKa-pHves}+1} + B \qquad \text{(AII1)}$$

where G_{DV} and G_{ves} correspond to the population of fluorescence molecules located in each respective compartment (ie the fluorescence observed when 100% of the molecules are in the fluorescent form ie under conditions where $H^+ << Ka$). The sum of these terms corresponds to the term G_t in Eq (1), hence

$$G_{ves} = G_t - G_{DV} \qquad \text{(AII2)}$$

The parameters pKa, G_t and B are determined from fitting a simple acid/base transition to the pH calibration data (Eq (1)). Additional information is required to remove the G_{DV} term from Eq (AII1) and (AII2). The fraction of the fluorescence intensity that arises from the DV population in the native sample (f_{DV}) can be estimated using independent microscopy measurements:

$$f_{DV} = \frac{F_{DV}}{F_{DV} + F_{ves}} = \frac{F_{DV}}{F_n - B} \qquad \text{(AII3)}$$

Solving Eq (AII3) for F_{DV} and equating this to the term describing the pH dependence of the DV population provides.

$$F_{DV} = f_{DV}\,(F_n - B) = \frac{G_{DV}}{10^{\,pKa - pH_{DV}} + 1} \qquad \text{(AII4)}$$

Solving Eq (AII4) for G_{DV} and substitution into Eq (AII1) and (AII2) provides the general solution to determining the pH of one compartment in the presence of a population existing in a secondary compartment.

$$pH_{DV} = pKa - log\left(\frac{G_t - (F_n - B)(1 - f_{DV})(1 + 10^{\,pKa - pH_{ves}})}{f_{DV}(F_n - B)} - 1\right) \qquad \text{(AII5)}$$