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Online Supplemental Material

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Kinetic analysis of photobleaching experiments

10 different FRAP and 5 different FLIP experiments were used for kinetic analysis to derive the dissociation constant k_{off} of GFP_3-hNup133 from kinetochores. The FRAP experiments can be fit by a simple model for hNup133 binding to kinetochores that assumes mass action kinetics to a single type of binding site. Since the experiments were performed in stable cell lines, we assume that the mitotic distribution of GFP_3-hNup133 is in steady state and that the fluorescently tagged protein reflects the behavior of its endogenous counterpart. The general kinetics for a steady state ligand (in this case GFP_3-Nup133) receptor (in this case unknown component of the kinetochore) interaction is expressed in Eq. 1:

$$[L]*[R]*k_{on} = [LR]*k_{off}$$

where [R] = free receptor, [L] = free ligand, [LR] = bound ligand, k_{on} = association constant, and k_{off} = dissociation constant.

The differential equation governing the change of bound ligand over time is expressed in Eq. 2:

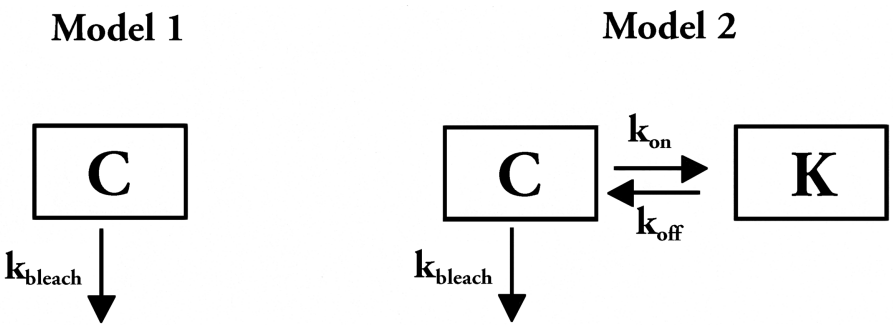
$$d[LR]/dt = k_{on}*[L]*[R] - k_{off}*[LR]$$

In steady state, R is constant. Assuming binding of GFP_3-hNup133 is not diffusion limited and that cytoplasmic GFP_3-hNup133 is in large excess to kinetochore-bound GFP_3-hNup133, we can also assume L to be constant in a FRAP experiment. This simplifies the solution of Eq. 2 to a simple single exponential expression (Eq. 3):

$$[LR]\ (t) = A*(1 - \exp(-k_{off}*t)) + LR_0$$

where $A = [L]*[R]/K_d - LR_0$, $K_d = k_{off}/k_{on}$, LR_0 = bound ligand at time zero after the bleach. LR_0 is introduced to allow incomplete bleaching of bound GFP_3-hNup133 right after the bleach. Eq. 3 could fit all FRAP experiments and gave a mean $k_{off} = 0.0058\ s^{-1} \pm 0.0026\ s^{-1}$.

To independently determine k_{off} from FLIP experiments, we designed a simple kinetic model using the SAAM II software (SAAM Institute). The model had two compartments representing the cytoplasm (C) and kinetochores (K):



Fluxes between C and K are governed by first order rate constants. Since FRAP experiments showed an immobile fraction of ~30% of GFP_3-hNup133 on kinetochores, 30% of K was set to be unexchangeable. Mean cytoplasmic and kinetochore fluorescence was measured as described for FRAP and transformed to total intensities by multiplication with the volumes measured for cytoplasm and kinetochores in a three-dimensional reconstruction of the entire cell (as described above). The rate constant k_{bleach} with which fluorescence was lost from C was determined to $0.0063 \pm 0.0008\ s^{-1}$ by fitting fluorescence depletion from the cytoplasm with the simple model 1 using the the generalized nonlinear least squares optimization procedure of the SAAM II software. This value was then fixed and used to fit depletion from kinetochores with model 2 to determine k_{off}. k_{on} could not be determined with confidence from our data because of the large cytoplasmic excess of GFP_3-hNup133 compared with the kinetochore-bound pool. Therefore, we fixed k_{on} to a value that allowed us to fit k_{off} for all experiment datasets and obtained a mean $k_{off} = 0.0054 \pm 0.0013\ s^{-1}$ in excellent agreement with the results of the FRAP experiments. It is interesting to note that the fits were worse when no immobile fraction of K was introduced, validating the result of the FRAP experiments. Mean residence times are given as the inverse of k_{off}.