

3D-iFRAP: A TECHNIQUE TO DETERMINE DISSOCIATION RATES OF PROTEINS IN REPRESSION COMPLEXES IN LIVING *DROSOPHILA*

Gabriela Ficz, Rainer Heintzmann¹, Bernd Rieger, and

Donna J. Arndt-Jovin

Dept. of Molecular Biology, Max Planck Institute for Biophysical Chemistry,
37070 Goettingen, Germany

¹Randall Division of Cell & Molecular Biophysics

King's College London, Guy's Campus, London SE1 1UL, U.K.

email: djovin@gwdg.de

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During *Drosophila* embryogenesis the Polycomb-group genes (PcG) are responsible for the maintenance of the spatially distinct repression of developmentally important regulators like the homeotic genes for up to ten cell divisions before terminal cell differentiation and have been called “cellular memory genes.” PcG gene products act in multimeric complexes that associate with specific sequences in the genome called Polycomb Repression Elements (PREs) and inhibit transcription of the associated genes. However, the composition, lifetime and structure of the complexes are not known. Our goal is to understand the mechanism by which long-term silencing of chromatin over many generations is compatible with the ability to modulate the transcriptional state.

Green fluorescent protein (GFP) fusion proteins of two PcG proteins, Pc and Ph, have been constructed and introduced into transgenic flies. Ph-GFP was shown to rescue the null phenotype of the *Ph* gene, thus demonstrating that the fusion protein can make a competent repression complex. We performed fluorescence bleaching and recovery studies (FRAP and a new technique, 3D- iFRAP) in living embryos and live larval tissues of these transgenic stocks. 3D-inverseFRAP is a 4D-imaging technique that consists of bleaching the whole nucleus except for a small region surrounding a fluorescent locus of interest, recording a time series of (7) confocal z sections for several hundred seconds after bleaching, tracking the fluorescent locus in 3 dimensions (3-D), alignment of the locus in 3-D and calculation of the spot intensity using a weighted region of interest. The decrease in fluorescence intensity, corrected for background and bleaching during recovery, was normalized to the prebleach value and these I_{norm} values were plotted for each time point and the curves fitted to a single exponential decay function which gives the dissociation rate of the complex. In embryos we found a single dissociation rate for Ph-GFP of $k_{\text{off}}=0.047/\text{s}$ with a high statistical confidence, and a similar value of $k_{\text{off}}=0.029/\text{s}$ for Pc-GFP. In larval tissue 15-20% of the loci exhibited residence times of up to 400 s, corresponding to a dissociation rate of $k_{\text{off}}=0.005/\text{s}$, that is, rates between two and ten times slower than the majority.

Combining these measurements with data on the diffusion constant of the free protein derived from FRAP experiments we were able to analyze individual complexes and determine the binding equilibria. Our data demonstrate that the mechanism of long-term silencing maintained over 14 cell generations is achieved by mass action equilibria of freely dissociable complexes that have residence times no longer than 10 minutes.