

## ■ MOLECULAR BIOLOGY

**Measuring *In Vivo* Binding**

Sprague BL, Muller F, Pego RL, Bungay PM, Stavreva DA, and McNally JG. Analysis of binding at a single spatially localized cluster of binding sites by fluorescence recovery after photobleaching.

*Biophys J* 91: 1169–91, 2006.

**F**or many years, an assortment of established biochemical techniques have provided *in vitro* estimates of the binding affinity of different proteins to cellular target molecules.

However, it has been impossible to determine how close these *in vitro* affinity measurements are to the actual *in vivo* affinities, simply because there have been no established methods for measuring binding affinities within live cells. Techniques for this are now becoming available due to advances in light microscopy and, interestingly, the first results reveal striking differences compared with the *in vitro* estimates.

One approach for measurement of *in vivo* binding is fluorescence recovery after photobleaching (FRAP). This technique is performed by photobleaching fluorescent molecules at a specified location in a cell, and then monitoring the rate at which the bleached molecules are replaced by unbleached ones. This recovery rate will be influenced by the rate at which the fluorescent molecule diffuses. In addition, if the fluorescent molecule binds to a relatively immobile substrate such as DNA, then the recovery rate will also be affected by the strength of that binding interaction. Tightly bound molecules will yield much slower FRAPs than weakly bound molecules.

To tease out the contribution of diffusion and binding from FRAP data, mathematical models are required that account for both processes. The equations describing a FRAP recovery include a term for diffusion, plus chemical kinetic terms for the on and off rates of binding to an immobile substrate. These equations can be used to predict FRAP recoveries, and therefore determine which combination of diffusion constant, on rate, and off rate will yield the best match to experimental FRAP data.

We have developed, analyzed, and applied such model equations to FRAP data for the glucocorticoid receptor, a transcription factor that resides in the cell nucleus after exposure to steroid hormone. In earlier work, we showed that binding of the glucocorticoid receptor to specific promoter sequences could be visualized in live cells containing an array of 200 tandemly repeated copies of the promoter and downstream reporter genes stably integrated into a mouse chromosome. A series of control experiments demonstrated that transcription occurs normally from this promoter array, which appears as a bright spot within a cell

nucleus containing a green fluorescent protein (GFP)-tagged glucocorticoid receptor.

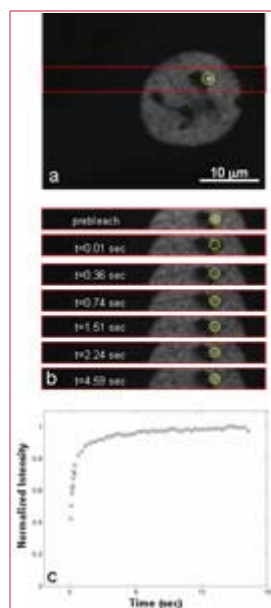
FRAP experiments performed at the promoter array will therefore yield recovery data containing information about the *in vivo* binding of the glucocorticoid receptor to the promoter sites. To estimate these *in vivo* binding parameters, we developed a mathematical model to account for FRAP at a spatially localized cluster of binding sites. The resultant partial differential equations were solved numerically using a finite element method, and then used to predict FRAP curves at the promoter array.

With this model, we obtained excellent fits of the experimental FRAP data using only a single, free parameter, namely the ratio of the on to off rates of binding at the promoter sites. This yielded an estimate for an *in vivo* binding constant of  $10^{-7}$  M, and an upper limit of 170 milliseconds for the residence time of a glucocorticoid receptor on a promoter. In stark contrast, *in vitro* binding estimates have yielded binding constants from  $10^{-8}$  to  $10^{-10}$  M and residence times of 90 minutes.

These striking differences almost certainly reflect, at least in part, the fact that the *in vitro* experiments were performed with naked DNA and purified glucocorticoid receptor, whereas *in vivo*, the promoter DNA is packaged as chromatin and the glucocorticoid receptor may be associated with a variety of cofactors that could influence its binding. However, since techniques for measurement of *in vivo* binding are still in their infancy, further work is needed to establish the validity of the mathematical models.

We have begun this process by incorporating a number of real-life features into the models, and then comparing the results of these more complex models to the results of simpler ones. This has shown that some assumptions have serious consequences, whereas others have insignificant effects and so in general can be ignored.

Although further computational tests will help refine the mathematical models, independent techniques for measuring *in vivo* binding will also be necessary to validate the FRAP estimates. Fortunately, several interesting complementary approaches, such as fluorescence correlation spectroscopy, are also being developed to estimate *in vivo* binding parameters. As these various techniques are refined and are shown to yield similar estimates for live-cell binding parameters, we will have moved a step closer to a new era of *in vivo* biochemistry by light microscopy.



**Figure 1.** A live cell nucleus containing hormone-induced green fluorescent protein–tagged glucocorticoid receptors (GFP-GR) and 200 tandemly repeated copies of a promoter array for GR (a). The array appears as a bright spot marked here by the yellow circle. In fluorescence recovery after photobleaching (FRAP) experiments, fluorescence is specifically bleached only inside the yellow circle, and then the rate at which fluorescence recovers there is monitored (b). Since the rate of fluorescence recovery is rapid, narrow strip images (red rectangle in part a above) are acquired on a confocal microscope, thereby reducing the scan time for acquisition of each image during the fluorescence recovery. Images at a few selected recovery time points are shown (b). Using all the collected time points, the average intensity inside the yellow circle is measured to generate a FRAP curve (c). The curve is normalized to one based on the initial intensity inside the yellow circle. Curves such as this can be fit to estimate values for the *in vivo* binding parameters of GR at a promoter.

**James G. McNally, PhD**

Facility Manager

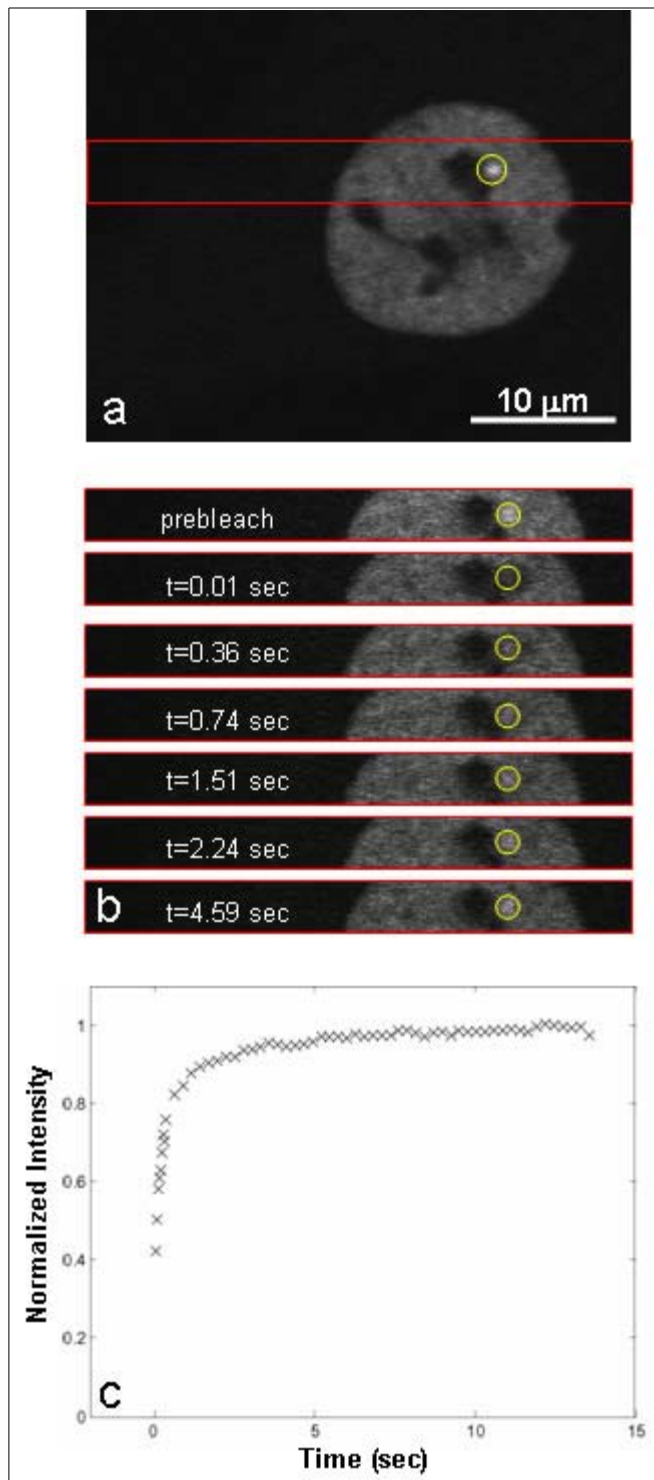
Laboratory of Receptor Biology and Gene Expression

NCI-Bethesda, Bldg. 41/Rm. C615

Tel: 301-402-0209

Fax: 301-496-4951

[mcnallyj@mail.nih.gov](mailto:mcnallyj@mail.nih.gov)



**Figure 1.** A live cell nucleus containing hormone-induced green fluorescent protein–tagged glucocorticoid receptors (GFP-GR) and 200 tandemly repeated copies of a promoter array for GR (*a*). The array appears as a bright spot marked here by the yellow circle. In fluorescence recovery after photobleaching (FRAP) experiments, fluorescence is specifically bleached only inside the yellow circle, and then the rate at which fluorescence recovers there is monitored (*b*). Since the rate of fluorescence recovery is rapid, narrow strip images (red rectangle in part *a* above) are acquired on a confocal microscope, thereby reducing the scan time for acquisition of each image during the fluorescence recovery. Images at a few selected recovery time points are

shown (*b*). Using all the collected time points, the average intensity inside the yellow circle is measured to generate a FRAP curve (*c*). The curve is normalized to one based on the initial intensity inside the yellow circle. Curves such as this can be fit to estimate values for the *in vivo* binding parameters of GR at a promoter.