TOWARDS THE VALIDATION OF IN-VIVO BINDING RATES MEASUREMENTS BY DIRECT QUANTITATIVE COMPARISON OF FCS AND FRAP

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Measurements of *in-vivo* binding reactions are essential to understand and quantify the molecular reactions that govern cellular processes and to provide the input data for the development of systems biology models. A number of groups have recently made these measurements using Fluorescence Recovery After Photobleaching (FRAP), but there is no "gold standard" to assess the accuracy of the resultant estimates.

As one step towards a gold standard, we have developed a new Fluorescence Correlation Spectroscopy (FCS) method to measure the in vivo bound fractions and residence times for molecules (such as transcription factors) that interact with an immobile substrate (such as DNA), since we find that existing methods for analyzing binding by FCS may be subject to error in their estimates of diffusion constants and bound fractions. We apply our new FCS method and also one of the published FRAP procedures [1] to measure diffusion and binding of three different molecules in the same cell line. We find that both FCS and FRAP yield quantitatively consistent estimates for the diffusion constant of unconjugated GFP. Both methods also yield quantitatively consistent estimates for the diffusion constant of GFP fused to just the dimerization domain of the transcription factor VBP (vitellogenin binding protein). Reassuringly, this estimated diffusion constant is consistent with the size of the GFP fusion protein. Finally, both FCS and FRAP also yield quantitatively consistent estimates for the estimated fraction of molecules bound to DNA when the VBP DNA binding domain is attached to the VBP dimerization domain. Notably, the FCS analysis provides an estimate of the VBP residence time on DNA while FRAP does not. We show that this limitation in the analysis of VBP by FRAP arises from the larger spot size that is typically used to perform the photobleach compared to the smaller diffraction-limited illumination volume used in FCS.

In sum, our results demonstrate that both FRAP and FCS can yield consistent estimates for diffusion constants and bound fractions, thereby providing some of the first indications that current procedures for measuring in vivo diffusion and binding are quantitatively accurate. Comparisons of other molecules will be necessary to assess the robustness of the present analysis, and in particular to permit a determination of whether both FRAP and FCS yield quantitatively consistent estimates for the residence times of binding.

[1] F.Mueller, P. Wach and J.G. McNally, "Evidence for a common mode of transcription factor interaction with chromatin as revealed by improved quantitative fluorescence recovery after photobleaching." *Biophys J.*, **94**, 3323-3339 (2008).