

# ConfoCor2-LSM 510

## Protein Complex Assembly Studied with FRAP and FCS

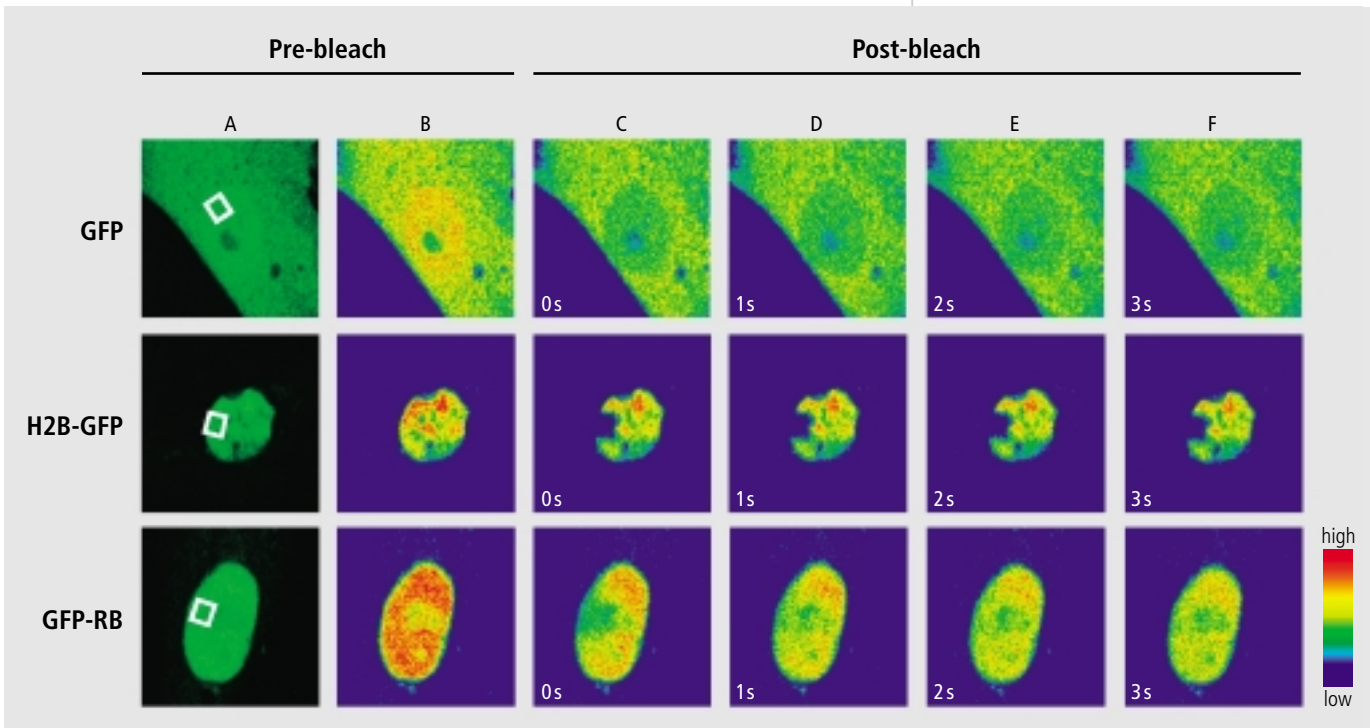


Fig.1 Description see page 4

The retinoblastoma tumor suppressor (RB) assembles multiprotein complexes to mediate transcriptional repression, cell cycle inhibition, and tumor suppression. We have employed a combination of fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) to show that a mutant RB allele refractory to phosphorylation assembles into large, relatively immobile complexes in living cells while the

wild type allele preferentially exists as a freely-diffusible monomer when phosphorylated.

The data show that FCS is a powerful tool for studying protein/protein interactions in living cells. In contrast to FRAP, FCS measurements can be performed in equilibrium. Furthermore, FCS allows distinction of different mobile populations. Thus, equilibrium binding constants within living cells can be obtained easily.

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**Fluorescence Correlation Microscopy  
 within Living Cells**



## Introduction

The retinoblastoma tumor suppressor (RB) functions as a negative regulator of the cell cycle by repressing the transcription of genes required for cell cycle progression. RB was first identified because of its genetic inactivation in retinoblastoma. RB has since been recognized as functionally inactivated in more than 60% of human tumors. Understanding the mechanism by which RB inhibits cellular proliferation is critical to understanding its role in tumor suppression.

RB is regulated by phosphorylation. When hypophosphorylated, RB is active and assembles multiprotein complexes to mediate transcriptional repression, cell cycle inhibition, and tumor suppression. In response to mitogenic factors or other proliferative signals, cyclin-dependent kinases phosphorylate RB resulting in RB inactivation and cell cycle progression (see Figs. 2 and 6).

Significant new techniques in analyzing molecular dynamics lend themselves to investigating complex behaviors within living cells. Here we have employed a combination of fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) to investigate the dynamics of RB and RB-assembled complexes in the nucleus of live mammalian cells. In control experiments free eGFP diffusion was observed.

## Materials and Methods

### Cell culture and transfection

Rat-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. Rat-1 cells were seeded on 25-mm coverslips and transfected with 4 µg of eGFP-WTRB or eGFP-MutRB expression vector by FuGENE 6 (Roche) according to the manufacturer's protocol. Thirty-six to forty-eight hours after transfection, coverslips were transferred to live-cell imaging chambers (Atto).

### Equipment

FRAP was performed on a Zeiss LSM 510 laser scanning confocal unit mated to a Zeiss Axiovert inverted microscope equipped with a C-Apochromat 63x 1.4 NA oil immersion objective. FCS was performed using the Zeiss ConfoCor2 - LSM 510 microscope with a C-Apochromat 40x 1.2 NA water immersion objective.

### FRAP Measurements

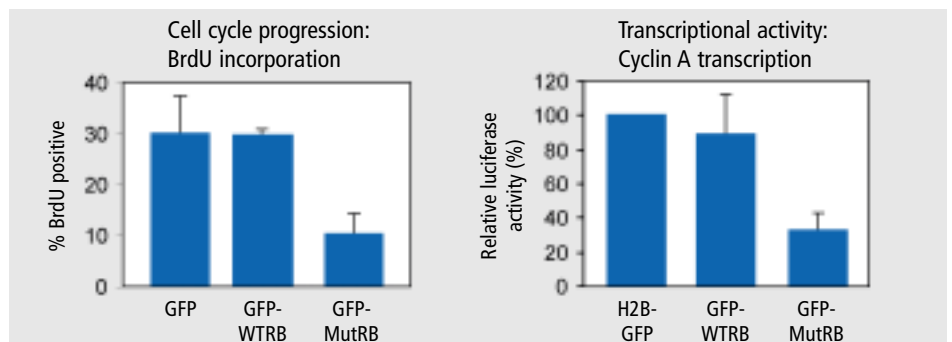
A 2.9 x 2.9 µm<sup>2</sup> area of the nucleus was photobleached for 0.688 seconds with 488-nm light from an argon laser at full intensity. Fluorescence intensity values of the bleached area and of a distal unbleached area of the nucleus of equal size were measured every 50 milliseconds

for 4 seconds following photobleaching. These values were compared to produce a relative fluorescence intensity to normalize for pre-bleach intensity. The mean time for 50% recovery ( $t_{1/2}$ ) was determined from recovery curves by regression analysis using SigmaPlot software.

### FCS Measurements

eGFP fluorescence in cell nuclei was excited with 488-nm light from an argon laser and detected through a pinhole with a diameter of 70 µm and a 505 nm bandpass filter. Auto-correlation curves were derived from fluctuations of fluorescence intensity measured over four-second intervals. 20 measurements were taken per nucleus and the corresponding average correlation curve was exported to the Microcal Origin software. These curves were fitted to a two component model of free diffusion in two dimensions to derive the translational diffusion time and number of molecules diffusing through the confocal volume with  $D_{fast}$  and  $D_{slow}$ .

*Fig.2 Wild-type RB (WTRB) is readily phosphorylated in living cells. When introduced into Rat-1 cells, the phosphorylated form is not capable of cell cycle inhibition (left) or transcriptional repression (right) similar to free eGFP and the histone-eGFP fusion protein (H2B-eGFP), respectively. In contrast, a mutant allele of RB that is refractory to phosphorylation, MutRB, potently inhibits cell cycle progression and transcription of Cyclin A.*



## Results

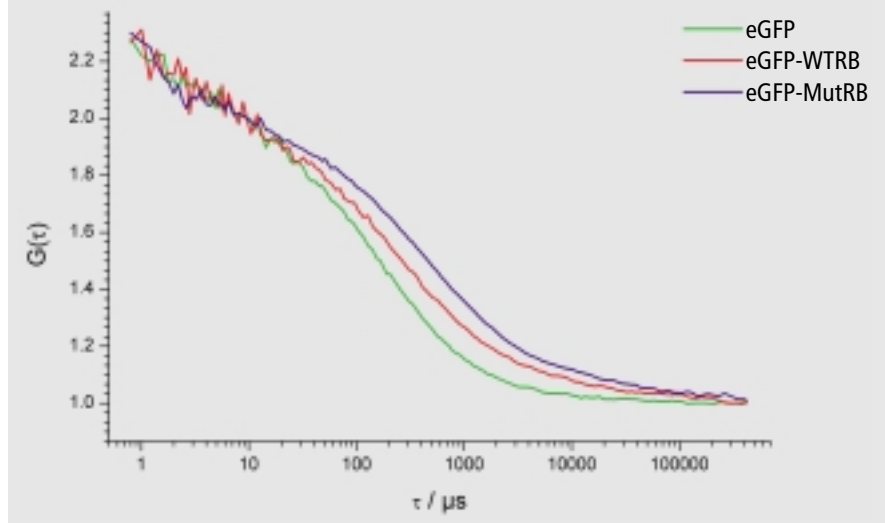
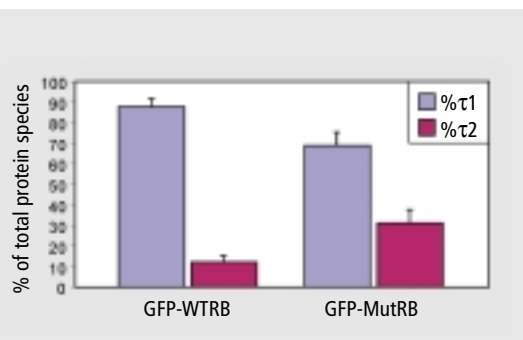
FRAP experiments show that eGFP recovers almost instantaneously following nuclear photobleaching with a  $t_{1/2}$  recovery value of less than 80 ms (see Fig. 1 and 3). This observation is consistent with a freely diffusible protein species.

Distinct from eGFP, we observed a recovery with significantly slower kinetics for eGFP-WTRB and eGFP-MutRB ( $t_{1/2}$  for WTRB = 220 ms and  $t_{1/2}$  for MutRB = 680 ms; see Fig. 3). These data indicate that the eGFP-fusion proteins are being assembled into relatively immobile complexes within living cells.

We then turned to FCS which allows for higher order dissection of molecular dynamics in live cells. FCS demonstrated that eGFP diffuses exclusively as a single species in the nucleus with a  $\tau_D$  of 205  $\mu$ s, again consistent with a freely diffusible protein species.

FCS revealed that both eGFP-RB alleles exist in two distinct states within the cell: the first as a highly mobile species with  $\tau_D$  of 285  $\mu$ s, and the second as a large, relatively immobile complex with  $\tau_D$  of 4000  $\mu$ s.

*Fig.5 FCS analysis of Rat-1 cells expressing the eGFP-RB constructs demonstrates that WTRB exists primarily as a monomeric species with diffusion time  $\tau_1$ . In contrast, greater than 30% of MutRB was found in large complexes with diffusion time  $\tau_2$ .  $\tau_1 = 280 \mu$ s and  $\tau_2 = 4000 \mu$ s.*



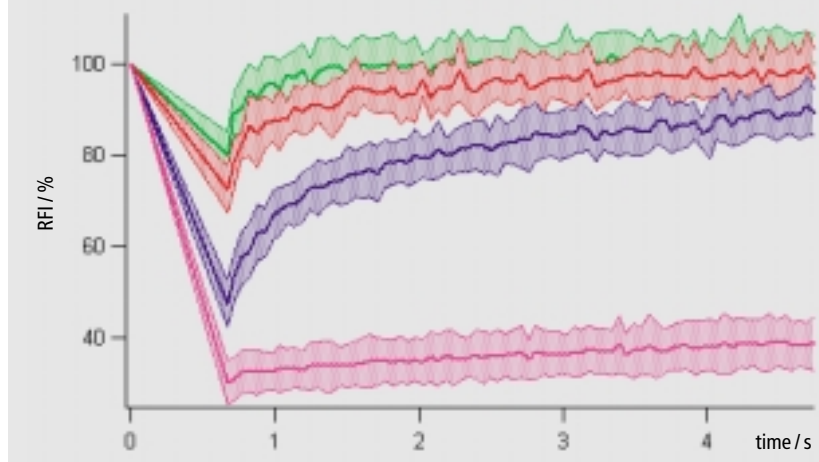
*Fig.4 Normalized autocorrelation curves describing the behavior of eGFP (green), eGFP-WTRB (red), and eGFP-MutRB (blue) in the nucleus of Rat-1 cells.*

A  $\tau_D$  of 285  $\mu$ s corresponds to a molecular weight of 80 kDa. This is comparable to the predicted molecular weight of monomeric eGFP-RB (93 kDa) and suggests that this rapidly diffusing species is the monomeric form of the eGFP-RB alleles.

A  $\tau_D$  of 4000  $\mu$ s for the large, relatively immobile species corresponds to the assembly of a very large complex in living cells by the eGFP-RB alleles. While both eGFP-RB alleles were found in large complexes, a 3-fold greater proportion of eGFP-MutRB was present in large complexes compared to eGFP-WTRB (see Fig. 5).

Many previous studies have used biochemical assays to understand how RB is regulated by phosphorylation. However, no prior study has investigated the phosphoregulation of RB in real time in living cells. Here, we were able to demonstrate for the first time by FCS that phosphorylated RB exists as a monomeric species in living cells while active, hypophosphorylated RB assembles multiprotein complexes to mediate transcriptional repression, cell cycle inhibition, and tumor suppression.

*Fig.3 Fluorescence recovery after photobleaching of eGFP (green), eGFP-WTRB (red), eGFP-MutRB (blue), and eGFP-H2B (lilac) in the nucleus of Rat-1 cells. Always the mean of at least 20 experiments +/- standard deviation is shown. RFI – Relative fluorescence intensity, given in percent.*



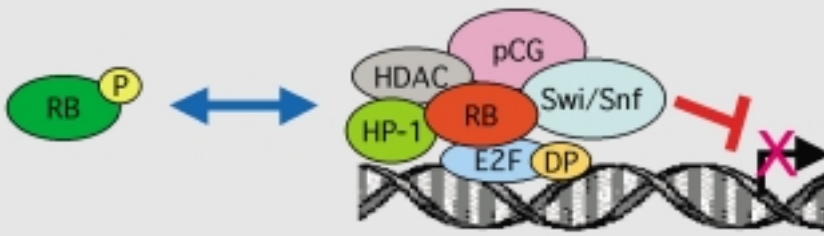


Fig.6 Model for the behavior of RB in living cells: Phosphorylated RB exists as a monomeric species in living cells while active, hypophosphorylated RB assembles multiprotein complexes to mediate transcriptional repression. (HDAC- histone deacetylase, HP1- heterochromatin protein 1, PCG – Polycomb group complex, SWI/SNF - SWI/SNF complex; E2F - RB associated proteins, DP – heterodimerization partner of E2F).

## Conclusion

Both FCS and FRAP are methods to investigate the mobility of fluorescent particles. From this data information about binding to other molecules or structures can be extracted. However, due to the different characteristics of these methods, the fields of application are different. FCS is used to elucidate the mobility of rather fast molecules such as free eGFP, and to obtain detailed information about the single populations that contribute to the fluorescent signal. With FRAP one can investigate slow moving particles such as large membrane proteins and measure the immobile fraction. Furthermore, it is possible to observe the connectivity of cellular compartments.

Taken together, FCS and FRAP are complementary, rather than competitive techniques. Using the ConfoCor2 - LSM 510 allows application of both FCS and FRAP. This gives the freedom to choose the appropriate method based on the cellular system and specific hypothesis being tested.

Fig.1 FRAP time series in the nucleus of Rat-1 cells expressing GFP, H2B-GFP (histone H2B), and GFP-RB. A  $2.9 \mu\text{m} \times 2.9 \mu\text{m}$  area of the nucleus was photobleached for 0.688 sec with 100 % transmission of 488 nm light from an argon laser. Images shown on the figure: pre-bleach (A and B, the white box in A represents the bleach region), immediately following the bleach (C), and at 1, 2, and 3 seconds post-bleach (D, E, and F, respectively). The colour code in images B-F indicates pixel intensity.

## Further reading

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