Fluorescence Recovery after Photobleaching (FRAP)

In these practicals, individual groups will study the dynamics of nuclear proteins in one of these model systems;

- **Proliferating cell nuclear antigen (PCNA) during DNA replication**, *Johan Slotman*

DNA replication and gene transcription have in common that both processes act on DNA, which requires the recruitment of chromatin remodelers to the active sites. These active sites can be visualizes by confocal microscopy as foci or speckles, allowing us to count the number, the shape and the size of those structure at any stage of replication or transcription (this will be done in the time lapse imaging practical). In this practical you will either measure the binding kinetics of PCNA in individual foci or study the DNA binding by ARs using fluorescence recovery after photobleaching (FRAP)[1]. In both studies active and inactive proteins are compared, which is essential for proper interpretation of the results.

INTRODUCTION:

The model systems

Role of PCNA in DNA replication

DNA replication occurs during the S-phase of the cell cycle (Figure 3). Proliferating cell nuclear antigen (PCNA) is a clamp that supports DNA replication by binding as a trimer around the DNA and serve as a scaffold. Many other supportive proteins, including chromatin remodeling factors, interact with DNA-bound PCNA to further support proper replication. Similar to the androgen receptor PCNA that binds to DNA during replication accumulates in tiny structures, termed PCNA foci that can be visualized by confocal microscopy (Figure 4). *Replication starts in euchromatin in early S-phase. In mid S-phase heterochromatin (mostly near the nuclear membrane and the nucleoli) is replicated. Finally in late S-phase, a few large PCNA foci remain. It is currently not clear what the function of those larger foci is.* Thus, PCNA foci change during progression through S-phase both in size and number as will be studied in the time lapse imaging practical.

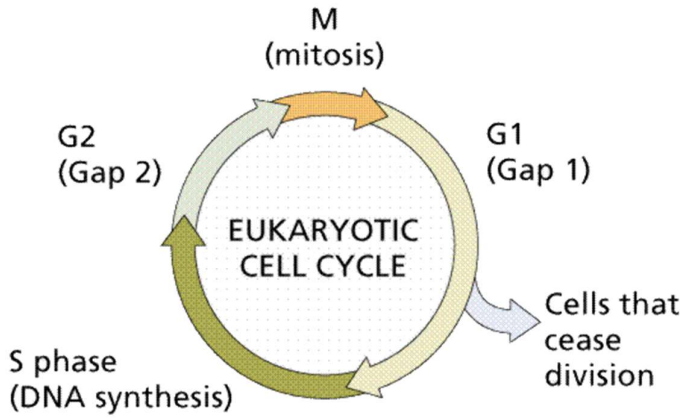


Figure 3. The cell cycle.

[1] note that in one of the other practicals FRAP will also be applied to focal adhesions, little structure at the cell membrane that are involved in cell migration; this is described in another protocol

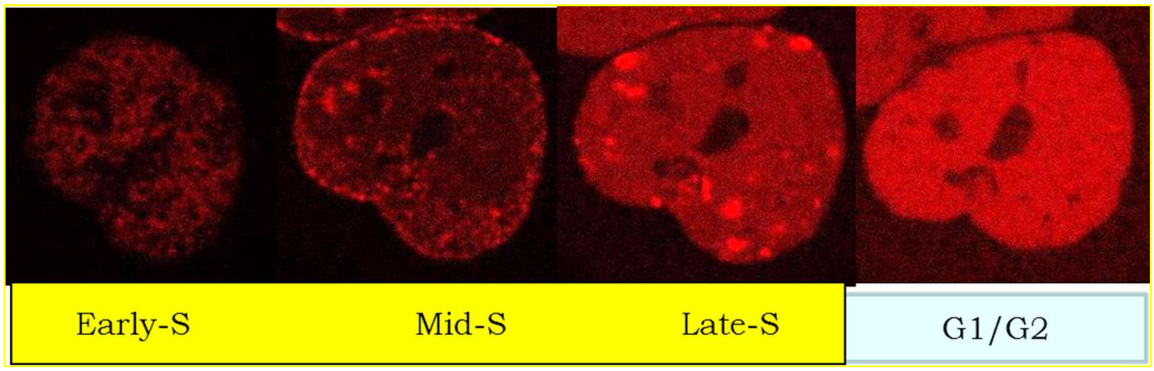


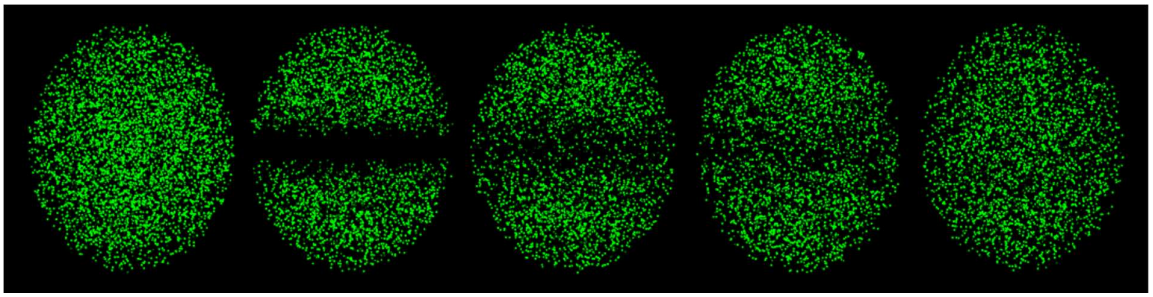
Figure 4. Confocal images of a cell expressing mCherry-tagged PCNA during the subsequent stages of S-phase an in G1 or G2 phase.

Here, we will compare the mobility of fluorescently-labeled PCNA in G1- or G2-phase with the mobility in the three subsequent stages of S-phase. The differences between the mobility of inactive PCNA (in G1 or G2) and active PCNA (in S-phase) reveals the DNA-binding behavior of PCNA.

Introduction on the FRAP approach

Fluorescent molecules emit light at a certain wavelength (e.g. green) after having absorbed light of lower excitation wavelength (and higher energy, e.g. blue). However, if the fluorescent molecule is exposed to a high intensity laser pulse, its capacity to fluoresce is destroyed. This process, called photo-bleaching, can be used in a powerful method called Fluorescence Recovery After Photobleaching (FRAP).

In a FRAP experiment (Figure 5 and 6), the scanning laser of a confocal microscope is used to irradiate a small area inside the cell at very high laser intensity for a short period of time and thereby locally photobleach the majority of the fluorescent molecules. The fluorescent molecules outside the small area that are mobile (e.g. because they diffuse) will move into the bleached area, resulting in the recovery of fluorescence after the bleach in the small area. The velocity at which fluorescence increases is a measure for the diffusion rate of the molecules. Immobile molecules do not move (e.g. because of DNA binding) and will not contribute to the recovery of fluorescence.



TIME →

Figure 5. Scheme of a typical FRAP experiment. From left to right: a cell nucleus containing fluorescently labelled molecules is imaged before bleaching a strip in the central region. After bleaching, mobile molecules move into the bleached region, leading to recovery of the fluorescent signal in the strip. Immobile molecules do not move and will not contribute to fluorescence recovery (see also Figure 6).

With FRAP we can determine the following kinetic parameters that give information on the way proteins perform their function in the cell:
• The **diffusion coefficient** of the molecule can be deduced from the speed at which fluorescence recovers to the final level (Figure 6, dark and light blue curve).
• When a **fraction of the molecules is immobile**, this fraction can be calculated from the incompleteness of the fluorescence recovery. When for instance half of the molecules is immobile, fluorescence will recover only to half the level it would if all molecules were mobile (Figure 6, red curve)
• The **average binding time** of molecules (time spent in the immobile state) can also be determined. After an initial rapid recovery by mobile molecules, molecules that bind shortly to DNA will release soon after the bleach and lead to a secondary recovery of fluorescence, (Figure 6, purple curve). The time it takes to reach complete recovery of fluorescence can then be used to determine the average binding time.

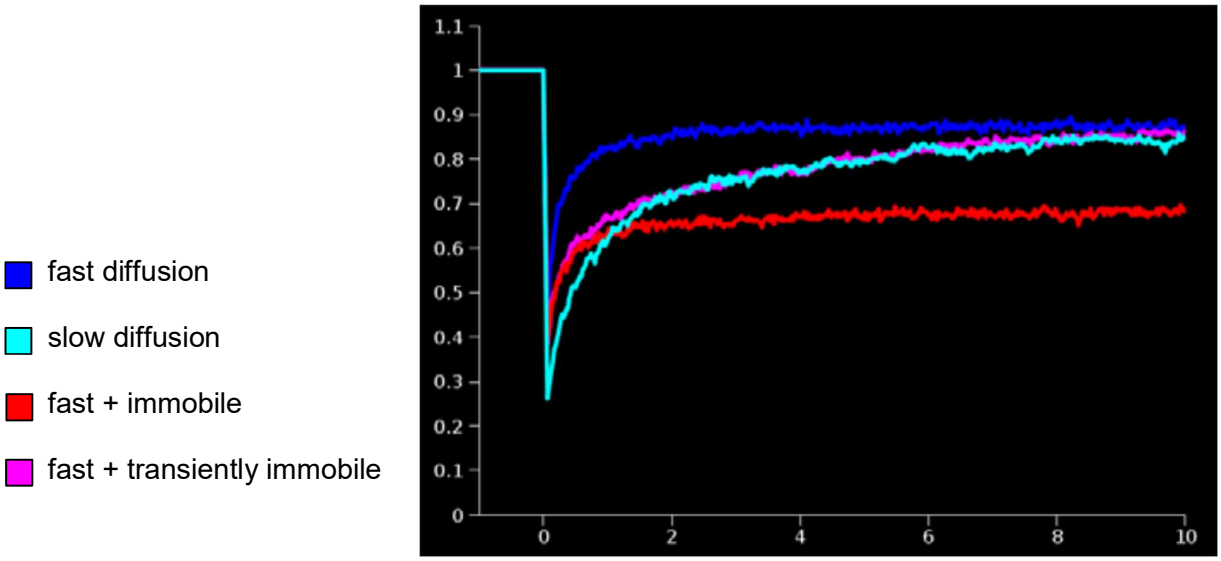


Figure 6. FRAP curves representing four different scenarios. Note that even in the case where all molecules are mobile, fluorescence does not recover to its initial level, since a considerable fraction of the molecules is bleached (~10%).

EXPERIMENTS:

You will receive detailed instructions on how to operate the microscope during the practical; minor changes to the protocol may be applied if necessary.

Strip-FRAP (general protocol):
Generate a FRAP curve for 5 cells of G-phase, early-S and Mid/Late-S

1. Select a nucleus with a moderate fluorescence (protein concentration) at a low zoom.
2. Zoom in on the selected nucleus (to a level that one pixel is 100x100 nm).
3. Rotate the (ellipsoid) nucleus so that the longest axis is vertical on the screen.
4. Record a sharp confocal image of the nucleus using averaging for reference.

5. Draw a horizontal strip (10 pixels thick) in the center of the nucleus spanning the entire width.
6. Set the number of images before bleaching (30=3S)
7. Set the number of bleach iterations (2)
8. Set the time lapse interval (100 ms)
9. Set the length of the time lapse (600=1min)
10. Start the time-lapse with bleaching.
11. Open an excel-sheet (only first time) and copy the recorded text file to the excel sheet.

For the Presentation:

1. Make an introduction for the biological system (PCNA)
2. Make an introduction about FRAP
3. Average the data of the 5 cells per phase
4. Make the normalized FRAP curves with the 3 normalization methods (see description below)
5. Combine the average data of the 3 phases with the second or the third normalization. Select the method that best shows what you want to present
6. Make a conclusion slide

FRAP ANALYSIS:
In order to determine an average curve form the individual curves of a specific experiment, the individual curves first have to be normalized. The general formula for normalization is:

$F(t) = (I_t - I_{min}) / (I_{max} - I_{min})$

where F is the normalized curve, t is time, I_t is the intensity value at time t, and I_{min} and I_{max} are the minimum and maximum intensity values.

1. Normalize each of the measured curves using:

    I_{min} = background intensity (discuss with the instructor how to image this)
    I_{max} = average intensity before the bleach.

2. Normalize each of the measured curves using:

    I_{min} = intensity immediately after the bleach
    I_{max} = average intensity before the bleach.

3. Normalize each of the measured curves using:

    I_{min} = intensity immediately after the bleach
    I_{max} = average intensity at complete recovery.

4. Make graphs of the individual curves (plot them in one graph per experimental condition or sample).

5. Calculate the average curve form the individual curves for each of the experimental conditions or sample.

6. Plot the average curves of the different conditions (samples) in one graph.