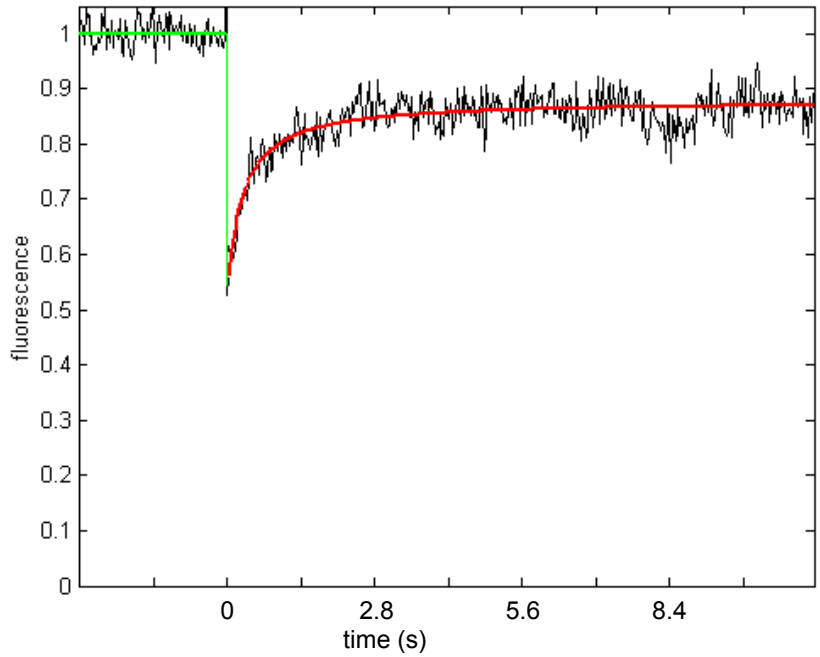

Figure 5: Representative FRAP curve of eYFP-C10HRas in the membrane of a tsA201 cell. Fitting using Eq. S1 & S2 resulted in a $t_{1/2}$ of 0.28±0.22 seconds and a mobile fraction $\alpha = 0.74 \pm 0.06$.

The FRAP experiments were conducted on the same microscope system used for single molecule imaging. During the experiments cells were kept in PBS at 22°C. For FRAP two beams were used: a highly focused, high intensity bleach-beam (400 kW/cm^2, diameter = 1.68 µm) for bleaching and a low intensity beam (2 W/cm^2, FWHM = 6.5 µm) for imaging. Illumination time and time between images were set to 3 ms and 28 ms respectively and kept constant during the experiment. FRAP curves were fit to the equation given by Feder et al (Feder et al., 1996):

$$F(t) = \frac{F_0 + F_\infty\left(\frac{t}{t_{1/2}}\right)}{1+\left(\frac{t}{t_{1/2}}\right)} \qquad \text{(S1)}$$

with a mobile fraction, α, defined as:

$$\alpha = \frac{F_\infty - F_0}{F^0 - F_0} \qquad \text{(S2)}$$

F_∞ is the fluorescence intensity for infinite times, F^0, the fluorescence intensity before bleaching and F_0 is the fluorescence intensity immediately after bleaching. The diffusion coefficient was derived from the half time for recovery, $t_{1/2}$, using:

$$D_{FRAP} = \beta \cdot \omega^2 / 4t_{1/2} \qquad \text{(S3)}$$

where ω represents the bleach beam radius (0.84 µm). The bleaching depth parameter β was 1.1 in our experiments, for a bleaching depth of 58 ± 11 %. (Axelrod et al., 1976;Yguerabide et al., 1982).

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Yguerabide,J., J.A.Schmidt, and E.E.Yguerabide. 1982. Lateral mobility in membranes as detected by fluorescence recovery after photobleaching. *Biophys. J.* 40:69-75.

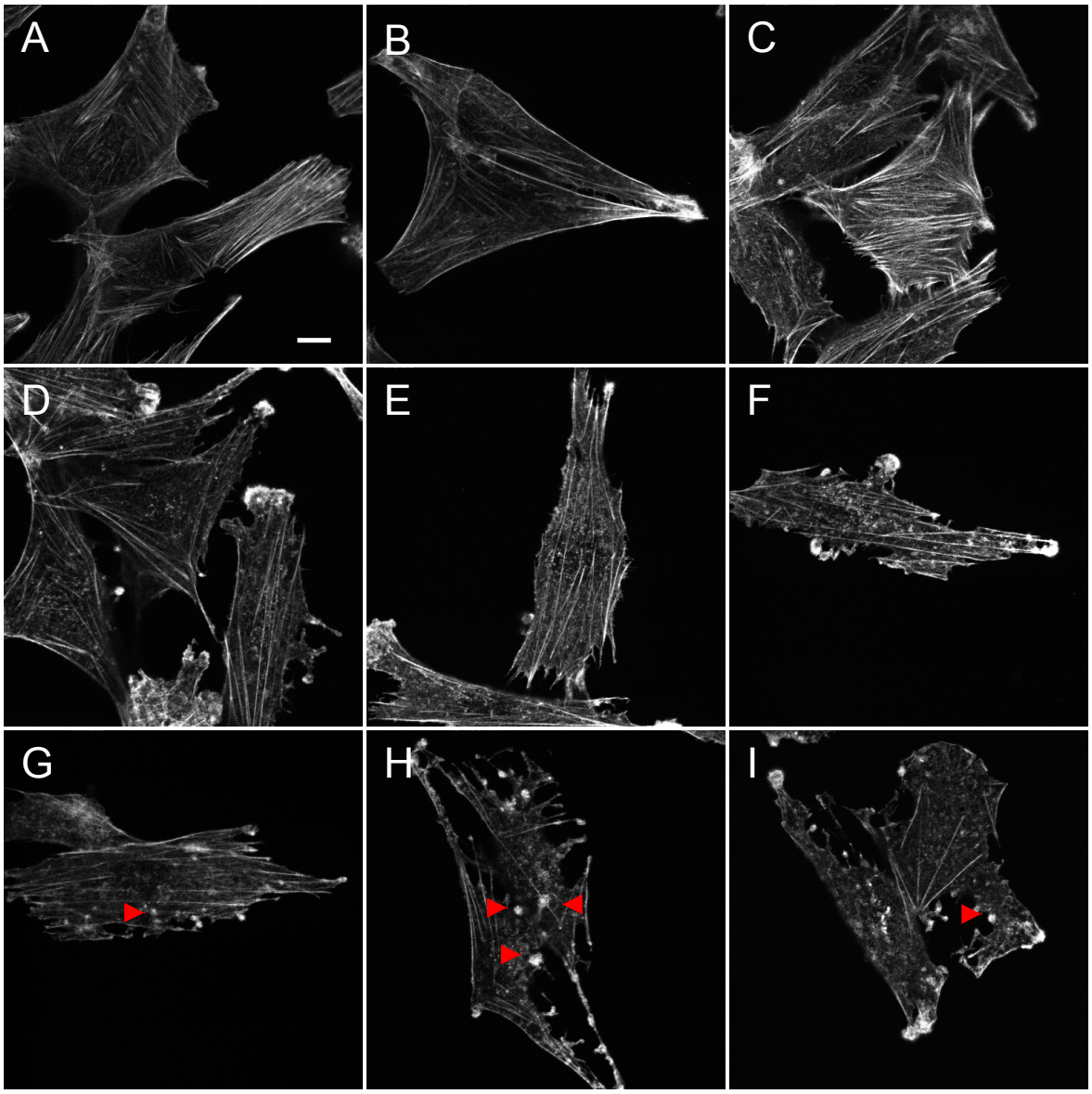

Figure 6: Changes in the organization of the actin cytoskeleton at various concentrations of latrunculin B.
3T3-A14 cells were incubated with 0.05% DMSO (A-C), 250 nM latrunculin B (D-F) or 500 nM latrunculin B for 30 minutes at 37°C/5% CO_2. After these treatments the cells were fixed and stained with Alexa568-phalloidin and imaged by scanning confocal microscopy. Treatment with 500 nM latrunculin B results in significant changes of the actin cytoskeleton. At this concentration small actin aggregates (arrowheads in G-H) coexist with cortical F-actin and less fibers are present. Scale bar: 10 μm.

Cholesterol depletion, membrane isolation and cholesterol quantification

Eight 100 mm dishes with 3T3-A14 cells were grown to ~80% confluency. Cells were incubated overnight in serum-free medium. In four dishes, the medium was replaced by serum-free medium containing 5 mM methyl-β-cyclodextrin and subsequently incubated 1 hour at 37°C/5% CO_2. The other four dishes with cells in serum-free medium were used as control. Cells were washed twice with PBS and detached by scraping in cold homogenization buffer: 20 mM Tris-HCl, pH 7.6, containing 10mM $MgCl_2$ and Protease Inhibitor Cocktail (cØmplete Mini EDTA free, Roche Diagnostics). 1 ml of buffer was used for 4 dishes. All of the subsequent procedures were carried out at 4 °C. The cell suspension was freeze-thawed (-80°C) 3 times and briefly sonicated with a tip-sonicator to further break up the cells (on ice). Total cell membranes (pellet) and cytosol (supernatant) were obtained by a 10 minute centrifugation in an Eppendorf centrifuge at 13000 rpm.

The pellet was resuspended in 0.1 ml of PBS + 0.5% NP40 (Non-idet). To obtain a completely resuspended total cell membrane extract, the pellet was sonicated in a bath sonicator (filled with ice-water) and regularly vortexed, to obtain a homogeneous solution. The cholesterol content of the solution was measured immediately after homogeneity was reached. The free cholesterol content was measured using the WACO free cholesterol-C kit (Waco Chemicals GmbH, Neuss, Germany), using the manufacturers instructions.

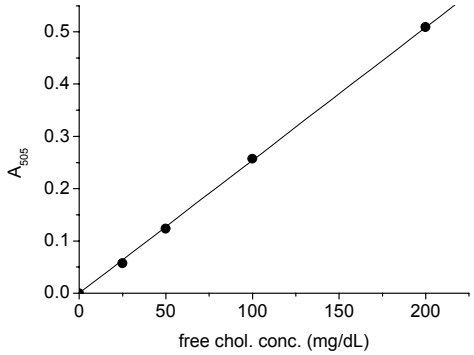

Figure 7: Calibration curve of absorption at 505 nm versus the free cholesterol content.

Table 2: cholesterol content of the total cell membrane preparations

Total cell membrane	A_{505}
Control	0.108
Cholesterol extracted	0.046

As the same weight of cell membrane material is used for both samples this means a reduction in cholesterol content of 57 ± 5 %.

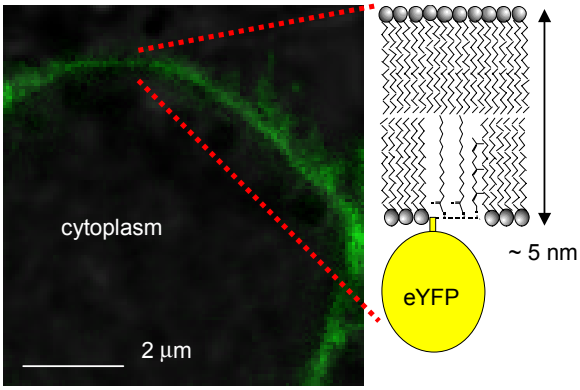

Figure 8: Confocal fluorescence image of a tsA201 cell showing clear plasma membrane localization of eYFP-C10HRas. Image was taken 48 hours after transfection of the cell with DNA encoding eYFP-C10HRas. The drawing on the right shows the schematic structure of the eYFP-C10HRas protein at the plasma membrane.