MEASURING THE MOBILITY OF SOLUBLE AND AGGREGATING PROTEIN USING FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP) IN NORMAL AND OSMOTICALLY STRESSED CELLS

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INTRODUCTION:
In FRAP experiments, fluorescent molecules are irreversibly bleached by a strong focused laser beam in a small area of the cell. Subsequent diffusion of non-bleached fluorescent molecules into the bleached area leads to a recovery of fluorescence, which is recorded at low laser power. The mobile fraction of fluorescent molecules and the rate of mobility can be deduced from FRAP studies. FRAP can be used to study protein localization, conformational changes, dynamics and interactions with other cellular components [1]. FRAP experiments in WP3 are used for studying cytoplasmic diffusion in cells and cell membranes.

MATERIALS
MEDIA

- LB medium per litre:
  - Tryptone 10 g
  - Yeast Extract 5 g
  - NaCl 5 g
  - For solid media add 14 g Agar per litre
  - For selective media add the following concentrations of antibiotics: ampicillin (100 mg/ml stock solution in dd H₂O), kanamycin (25 mg/ml stock solution in dd H₂O), chloramphenicol (25 mg/ml in EtOH). Ampicillin and kanamycin are sterile filtered through 0.2 µm filter pore size. All antibiotics are aliquoted in 500 µL and kept at -20 °C.
  - All antibiotics are provided by Sigma-Aldrich Company Ltd. (Germany).

- M9 minimal medium: 200 mL M9 salts without NaCl, 2 mL of 1 M MgSO₄, 0.1 mL 1M CaCl₂, 20 mL 20% glucose, add water to 1 L.
  - M9 salts without NaCl: 64 g/L of Na₂HPO₄ 7H₂O, 15 g/L KH₂PO₄, 5 g/L NH₄Cl, add ddH₂O to 1L; autoclaved and stored at room temperature
  - 1 M MgSO₄: MgSO₄ dissolved in ddH₂O and autoclaved
  - 1M CaCl₂: CaCl₂ dissolved in ddH₂O and autoclaved
  - 20% glucose: dissolved in ddH₂O and autoclaved; Note, autoclave glucose solution separately.
REAGENTS AND STOCK SOLUTIONS:
- 1000x stock of IPTG: 400 mM dissolved in ddH$_2$O and sterile filtered through 0.2 µm filter pore size
- 6M NaCl dissolved in ddH$_2$O and sterile filtered through 0.2 µm filter pore size
- 5 M stock solution of proline or glycine-betaine dissolved in ddH$_2$O and sterile filtered through 0.2 µm sterile filter

PROCEDURE:
SAMPLE PREPARATION FOR FRAP:
1. Freshly transform E.coli competent cells with 1 µL of the plasmid (here the pGEX-GFP-20Q (soluble protein) or pGEX-GFP-53Q (aggregating protein)). Transformation protocol: 45 min on ice, heat shock at 42 °C for 45 sec, 2 min on ice, and transfer into 1 mL pre-warmed LB medium and culture for 1 hr at 37 °C at 900 rpm. Plate on a LB-agar plate with corresponding antibiotic (here 100 µg/ml ampicillin)
2. Overnight culture: Inoculate a single colony into 5 ml LB medium supplemented with antibiotic (here 100 µg/ml ampicillin) and incubate et 30 °C at 220 rpm.
3. On the next day, gently spin down the cells at 2000 rpm in a table top centrifuge and resuspend the cells in 50 ml fresh LB medium containing the antibiotic (here 100 µg/ml ampicillin). Grow at 37 °C at 220 rpm till OD600 ~ 0.4; spin down the cells at 300 rpm in a sterile falcon tube in a table top centrifuge and resuspended in the same volume of M9 minimal medium
4. Split the culture in 5 mL and transfer each alquot in 50 mL sterile falcon tubes. Add 0.4 mM IPTG. (1) One is the control culture and add to it 50 mM NaCl from the sterile stock. (2) stressed cells: add 300 mM NaCl, (3) with secondary osmolyte proline: add 300 mM NaCl and 20 mM proline, (4) with secondary osmolyte glycine-betaine: add 300 mM NaCl and 20 mM glycine-betaine. Note, the same conditions can be used for any other secondary osmolyte, e.g., inositol, taurine, pipecolate. Grow the cells at 37 °C 220 rpm for three hours. Alternatively, the osmotic stress (with NaCl with or without secondary osmolytes) can be done also at different time points post-induction (0, 120 and 180 min).
5. For FRAP measurements approximately 4 µL of bacterial suspension is placed on a glass slide and covered with a cover slip precoated with concanavalin A (Sigma, Germany). All measurements were done at 37° ± 1°C. The concanavalin A adhesion does not have any effect on the mobility measurements with FRAP since similar results were obtained with uncoated cover slips.

FLUORESCENCE MICROSCOPY:
The effective diffusion coefficients and the mobile fraction of the GFP-tagged proteins are measured on APPLIED PRECISION DeltaVision RT Life Cell Imaging System equipped with a 100x oil immersion objective with a numerical aperture of 1.25 and an inbuilt laser beam is used for photobleaching. 3 prebleach images are obtained; the sample is then fired with single 100-ms bleach pulse with 20% laser power and then a stream of images are acquired during the fluorescence recovery. The exposure time is fixed at 0.05 sec/frame. For fluorescence recoveries, a continuous set of images is obtained with no dead time in between. The softWoRx software is used to analyse the FRAP data.
References