Chapter 3

On the mobility, membrane location and functionality of mechanosensitive channels in *Escherichia coli*

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Published in *Scientific Reports*, 2016

Abstract

Bacterial mechanosensitive channels protect cells from structural damage during hypoosmotic shock. MscS, MscL and MscK are the most abundant channels in *E. coli* and arguably the most important ones in osmoregulation. By combining physiological assays with quantitative photo-activated localization microscopy (qPALM), we find an almost linear relationship between channel abundance and cell survival. A minimum of 100 MscL (or MscS) channels is needed for protection when a single type of channel is expressed. Under native-like conditions MscL, MscS as well as MscK distribute homogeneously over the cytoplasmic membrane and the lateral diffusion of the channels is in accordance with their relative protein mass. However, we observe cluster formation and a reduced mobility of MscL when the majority of the subunits of the pentameric channel contain the fluorescent mEos3.2 protein. These data provide new insights into the quantitative biology of mechanosensitive channels and emphasizes the need for care in analyzing protein complexes even when the fluorescent tag has been optimized for monomeric behavior.

JvdB performed the molecular biology and microscopy studies; HG and SM the molecular biology and physiology studies; AR performed the patch clamp experiments; JvdB, HG, SM and BP designed the experiments, carried out the data analysis and wrote the manuscript.
Introduction

Biological membranes are complex structures allowing for the co-existence of single proteins and multiprotein assemblies in a milieu made of many different classes of phospholipids. Controlled localization of membrane proteins can be an essential aspect of cell growth and cell division [1]. Some proteins are present in the concave regions of the cell membrane, whereas other distribute homogenously over the lipid bilayer [2, 3]. Membrane proteins can be organized in specific lipid domains and associate to form supramolecular structures [4]. Protein mobility has biological importance in membrane organization, protein-protein interactions, energy conversion, signaling, cell division, chemotaxis, and osmotaxis [5-8]. Here, we focus on understanding the localization, mobility and role in osmoprotection of the multi-subunit complexes that make up the families of mechanosensitive (MS) channels.

*E. coli* has seven MS channels, MscL (~75 kDa) being the most extensively studied one and the main contributor to cell survival during a hypoosmotic shock [9, 10]. The other six channels belong to the MscS family, of which MscS (~210 kDa) and MscK (~900 kDa) are considered the major ones among the homologues [11, 12]. When cells face a hypoosmotic shock MS channels act as emergency valves and they release solutes in a non-specific manner enabling a rapid decrease in osmolyte concentration and, consequently, in the osmotic driving force for water entry [13]. Mechanosensitive channels are interpreters of membrane tension, caused by changes in water movement across the membrane, and they gate in response to this signal. The interaction of MS channels with phospholipids has been studied using electrophysiology, crystallographic, spectroscopic and mutational analysis [14-26]. Despite great advances at the structural and functional level there are contrasting reports about the location of the channels in the membrane [27]. It is unclear if channels are organized in clusters [28] and how many channels are required for osmoprotection.

To understand the dynamics of MS channels we combined single-molecule techniques with physiological assays. Photo-activated localization microscopy (PALM) and single-molecule tracking (SMT) were used to study the membrane localization and mobility of channels, which were fused to the photo-switchable fluorescent protein mEos3.2. We found that MscS, MscL and MscK are mobile along the cell but that excessive overexpression of MscL-mEos3.2 led to cluster formation and retarded mobility, yet the channels are fully functional as shown by electrophysiology studies. Co-expression of wild type subunits with those
tagged with mEos3.2 reduced the clustering, and thus we attribute the clustering to self-association of the fluorescent moieties rather than MscL itself. By combining quantitative PALM (qPALM) with a downshock assay we correlate the average channel numbers per cell with survivability after a rapid 0.3 M NaCl hypoosmotic shock. We find that around 100 plasmid-derived MscL channels confer full protection in a strain deleted for all chromosomal genes of MS channels. For MscS we could not achieve a condition to provide full osmoprotection but cell survival correlated with an increasing number of channels.

Materials and Methods

Strains and plasmids

E. coli strains MG1655 and MJF641 (Δ7) were used for our studies. Patch clamp recordings were done in E. coli MJF429 and MJF453 and maturation analysis in E. coli MC1061. All strains are described in Table 1. To create the channel fusions in pTRC, all pTRC-MscX vectors (X refers to any of the MS channel proteins) were linearized by XhoI digestion. The unique XhoI site is at the end of each mscX gene and immediately before the DNA encoding the poly-His tag. mEos3.2 was amplified from the pBAD-mEos3.2 plasmid by Polymerase Chain reaction (PCR). Primers to amplify mEos3.2 had a 15 bp sequence homologous to both extremes of the linearized pTRC-MscX, allowing the ligation of the mEos3.2 PCR products into the pTRC sequence. The ligation step was performed using the CloneEZ® PCR Cloning Kit (GenScript). For cloning the fusion constructs in pBAD the USER™ enzyme (Uracil-Specific Excision Reagent Enzyme) was used. In this approach primers are designed to have a selected T nucleotide replaced by a U (see Table 3). Amplified sequences included the backbone (pBAD), genes (mscS, mscL and mscK) and the fluorescent protein (mEos3.2). All plasmids and primers are listed Tables 2 and 3.

Table 1: E. coli strains used in this study.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJF641</td>
<td>Frag1, DkefA(mscK)::kan, DyjeP, DyggB(mscS), DF786(ybiO), mscL::cm, ybdG::Apr, F343(ynaI), ycjM::Tn10</td>
<td>[11]</td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12, F′.ilvG-rfb-50 rph-1</td>
<td>[29]</td>
</tr>
<tr>
<td>MJF429</td>
<td>Frag1, DyggB(mscS), DkefA(mscK)::kan</td>
<td>[19]</td>
</tr>
<tr>
<td>MJF453</td>
<td>Frag1, DkefA(mscK)::kan, DmscL::Cm</td>
<td>[19]</td>
</tr>
<tr>
<td>MC1061</td>
<td>K-12, F′.λ− Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 e14− merA0 relA1 rpsL150(StrK) spoT1 merB1 hsdR2(r-m+)</td>
<td>[30]</td>
</tr>
</tbody>
</table>
Table 2: Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRC-MscS</td>
<td>NcoI-HindIII mscS-bearing fragment cloned into NcoI-HindIII sites of pTRC99a plasmid backbone</td>
<td>[20]</td>
</tr>
<tr>
<td>pTRC-MscL</td>
<td>NcoI-HindIII mscL-bearing fragment cloned into the pTRC-MscS backbone</td>
<td>This study</td>
</tr>
<tr>
<td>pTRC-MscK</td>
<td>NcoI-HindIII mscK-bearing fragment cloned into NcoI-HindIII sites of pTRC99a plasmid backbone</td>
<td>Kind gift from T. Rasmussen</td>
</tr>
<tr>
<td>pTRC-MscS-mEos.3.2</td>
<td>mEos3.2 fragment with 15 bp homology to both extremes of the linearized pTRC-MscS cut by XhoI. The cloning tagged mEos3.2 at the C-terminal sequence of MscS</td>
<td>This study</td>
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<tr>
<td>pTRC-MscL-mEos3.2</td>
<td>mEos3.2 fragment with 15 bp homology to both extremes of the linearized pTRC-MscL cut by XhoI. The cloning tagged mEos3.2 at the C-terminal sequence of MscL</td>
<td>This study</td>
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<tr>
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<td>mEos3.2 fragment with 15 bp homology to both extremes of the linearized pTRC-MscK cut by XhoI. The cloning tagged mEos3.2 at the C-terminal sequence of MscK</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD-MscS-mEos3.2</td>
<td>NcoI-XmaI mEos3.2 fragment cloned into NcoI-XmaI sites of pBAD plasmid backbone</td>
<td>Kind gift from A. Robinson [31]</td>
</tr>
<tr>
<td>pBAD-cLIC-GFP</td>
<td>SmaI-XbaI fragment of pBAD-cLIC and an XbaI-digested PCR product holding an NcoI-free sequence coding for EGFP</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD-MscS-mEos3.2</td>
<td>mscS and mEos3.2 genes with 15 bp homology to each other and to the pBAD vector were cloned with a linker (GGENLYFQ) separating mscS and mEos3.2</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD-MscL-mEos3.2</td>
<td>mscL and mEos3.2 genes with 15 bp homology to each other and to the pBAD vector were cloned with a linker (GGENLYFQ) separating mscL and mEos3.2</td>
<td>This study</td>
</tr>
<tr>
<td>pACYC-LacY-mEos3.2</td>
<td>NcoI and BamHI LacY-mEos3.2 fragment cloned into pACYC backbone</td>
<td>This study</td>
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Table 3: Primers used in this study.

<table>
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<th>Primer</th>
<th>Template for PCR</th>
<th>Primer sequence (5' to 3')</th>
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<td>pBAD-mEos3.2</td>
<td>GAAGACAAAGCTGCGATGGGAAGTTCAGATT</td>
</tr>
<tr>
<td>mscS Fwd</td>
<td>pTRC-MscS</td>
<td>ATTAACCAUGGAAAGATTTGATTGCGATAGC</td>
</tr>
<tr>
<td>mscL Fwd</td>
<td>pTRC-MscL</td>
<td>ATTAACCAUGGACATTAAAGAATTTCGCAGATT</td>
</tr>
<tr>
<td>mscK Fwd</td>
<td>pTRC-MscK</td>
<td>ATTAACACAGCATATGATGTTGCGATAGC</td>
</tr>
<tr>
<td>mEos3.2 Fwd</td>
<td>pBAD-mEos3.2</td>
<td>AGGGGAAAUTTATATATATATATATATATAT</td>
</tr>
<tr>
<td>pBAD Fwd</td>
<td>pBAD-cLIC_GFP</td>
<td>ACCACACCAUCATCATCATACATCAGTCCCTGCGAATAGC</td>
</tr>
<tr>
<td>mEos3.2 Rev</td>
<td>pBAD-mEos3.2</td>
<td>GTGTTGTTGTTGTTGCGCCCTGCGAATAGC</td>
</tr>
<tr>
<td>mscS Rev</td>
<td>pTRC-MscS</td>
<td>ATTTTTCCUCCCCGACGCTTTGCTTCTTAC</td>
</tr>
<tr>
<td>mscL Rev</td>
<td>pTRC-MscL</td>
<td>ATTTTTCCUCCCCGACGCTTTGCTTCTTAC</td>
</tr>
<tr>
<td>mscK Rev</td>
<td>pTRC-MscK</td>
<td>ATTTTTCCUCCCCGACGCTTTGCTTCTTAC</td>
</tr>
<tr>
<td>mEos3.2 Rev</td>
<td>pBAD-mEos3.2</td>
<td>ATGTGTGTGTGTGCATCAGTGGCATTGCGAATAGC</td>
</tr>
<tr>
<td>pBAD Rev</td>
<td>pBAD-cLIC_GFP</td>
<td>ATGTGTGTGTGTGCATCAGTGGCATTGCGAATAGC</td>
</tr>
</tbody>
</table>

*For amplification of mEos3.2 for pTRC-MscX-mEos3.2
b*For amplification of mEos3.2 for pBAD-MscX-mEos3.2
**Growth conditions**
Transformed cells were grown overnight either in EZ rich defined medium (Teknova) supplemented with 0.2% (w/v) glycerol or Luria-Bertani (LB) medium in the presence of 50 or 100 µg/mL ampicillin. Cultures were incubated at 37 ºC under continuous shaking at 200 rpm and measured in exponential growth phase (OD_{600} ~ 0.3-0.5) unless otherwise stated.

**Electrophysiology**
Patch clamp recordings were conducted on membrane patches derived from giant protoplasts using the strain MJF429 (ΔmscS, ΔmscK) transformed with pTRC-MscS or pBAD-MscS-mEos3.2 and MJF453 (ΔmscL, ΔmscK) with pBAD-MscL-mEos3.2 plasmids. The cultures were induced with 1mM IPTG for 15 min (pTRC-MscS) or 0.5% L-arabinose for 65 min (pBAD-MscS-mEos3.2 and pBAD-MscL-mEos3.2) before protoplast formation. Excised, inside-out patches were analyzed at membrane potential of -20 mV with pipette and bath solutions containing 200 mM KCl, 90 mM MgCl₂, 10 mM CaCl₂, and 5 mM HEPES buffer at pH 7. All data were acquired at a sampling rate of 50 kHz with 5-kHz filtration using an AxoPatch 200B amplifier and pClamp software (Molecular Devices). The pressure threshold for activation is shown as the pressure ratio between MscL and MscS (P_L : P_S).

**Osmotic downshock assay**
*E. coli* MJF641 harboring pTRC-MscS or pTRC-MscL was grown overnight at 37 ºC in LB + 50 µg/mL ampicillin. The next day cultures were diluted to OD_{650} = 0.05 in 10 mL pre-warmed LB and incubated until OD_{650} = 0.4. Cultures were 10x diluted into a high-salt medium (LB + 0.3 M NaCl) and 0.3 mM IPTG was added when OD_{650} reached ~ 0.2. At OD_{650} = 0.3 an osmotic downshock was applied by diluting the cells 1:20 into LB medium (downshock) or into LB medium + 0.3 M NaCl (control). After 10 min of incubation at 37 ºC serial dilutions were made (10^{-1} to 10^{-5}) and 5 µL of each dilution was spotted in quadruplicate onto LB-agar plates (shock) or LB-agar plates containing additional 0.3 M NaCl salt (control). Survival was determined by comparing colony forming units on shock and control agar plates after overnight incubation at 37 ºC.

**Survival versus channel number**
Cultures of *E. coli* MJF641 harboring pBAD-MscS-mEos3.2 or pBAD-MscL-mEos3.2 were grown overnight in LB plus 100 µg/mL ampicillin at 37 ºC. The next morning a 1:100 dilution was made in 10 mL pre-warmed LB. When an OD_{650} = 0.4 was reached cultures were diluted 1:40 into 20 mL LB + 0.3 M NaCl to adapt to high salt, and the culture incubated until OD_{650} = 0.08. At
this point freshly prepared L-arabinose was added at final concentrations of 0.01%, 0.1%, 0.25%, 0.5% and 1% (w/v). After 1 h induction (optical density ~ 0.3 - 0.4), an osmotic downshock was applied by rapidly pipetting 0.5 mL of the cells into 10 mL LB (shock) and 10 mL LB + 0.3 M NaCl (control). Cells were incubated for 10 min at 37 °C prior to serial dilution and plating onto LB-agar or LB-agar + 0.3 M NaCl plates (described above). The survival of uninduced cells was also assessed. Concomitantly with the shock step, the remaining culture of cells grown in LB + 0.3 M NaCl was collected by centrifugation (~ 15 mL) for qPALM or filtration (for future downshock) and washed twice in PBS high salt (PBS adjusted with NaCl to the same osmolarity as LB to avoid downshock). Cells in PBS high salt were incubated at 37 °C for 5 h prior channel quantification with qPALM, to complete maturation of mEos3.2. At this point cells were also tested for survivability by dilution into PBS (shock) or high-salt PBS (control), after 10 minutes they were serially diluted in the same buffer and plated onto LB-agar or LB-agar + 0.3 M NaCl plates.

**Sample preparation for microscopy**

Coverslips (Carl Roth, LH26.1) were cleaned with 5 M KOH in a sonication bath, plasma-cleaned for 10 min and coated with 2% (v/v) (3-Aminopropyl)trimethoxysilane (Aldrich) in acetone for 15 min. Cells were grown as described above and prepared for image acquisition in exponential growth phase (OD\textsubscript{600} of 0.2-0.4). Cells were immobilized on coated coverslip with a second cleaned coverslip on top to prevent evaporation.

**PALM setup**

For single-molecule imaging a home-built inverted microscope based on an Olympus IX-81 microscope with a high numerical aperture objective (100 X, NA = 1.49, oil immersion, Olympus, UApo) was used. Solid-state lasers were from Coherent (Santa Clara, USA): 405 nm (Cube, 100 mW) and 561 nm (Sapphire 561, 100 mW). Laser beams were collimated with lenses and combined using dichroic mirrors. Laser power for mEos3.2 readout (561 nm) was set to ~1 kW/cm\textsuperscript{2}. The power for mEos3.2 activation (405 nm) was adjusted for each experiment based on labeling density. Imaging was performed in semi-TIRF and fluorescence was recorded using an electron multiplying charge coupled device (EM-CCD camera) from Hamamatsu, Japan, model C9100-13.

**PALM data acquisition and analysis**

To activate mEos3.2 molecules a pulsed laser was used for illumination. To detect single molecules typically 150-250 cycles of photoactivation were repeated with each cycle consisting of 20 frames. The first frame was used to switch mEos3.2 from a green to a red state, using a UV laser (405 nm). The
following 19 frames were used to readout the activated fluorescent proteins in the red channel (561 nm). Usually the read-out laser photobleaches all activated mEos3.2 molecules during these 19 frames so that the next cycle of photoactivation can be started. The exposure time of each frame was set to 31 ms. For qPALM up to 800 cycles were recorded to ensure that every mEos3.2 molecule was activated during the time of image acquisition. All measurements were carried out at 22 °C. The acquired movies were analyzed with a home-written ImageJ plugin. In the reconstructed images each fluorescent molecule was represented as a single spot at its determined coordinates with a brightness that corresponds to the localization accuracy.

**Single-molecule tracking**
The PALM data from MS channel fusions with mEos3.2 can be used to determine the diffusion constant $D$. A home-written ImageJ plugin was used to detect two fluorescent proteins in two consecutive frames that are in close proximity to each other (up to 320 nm), which resulted in trajectories of various lengths. The diffusion constant was calculated from the mean square displacement (MSD) by averaging ~10,000 trajectories that had a minimal length of 5 frames. The MSD curves were linearly fitted for points in the range of 0.03 to 0.25 s and diffusion coefficient $D$ was calculated assuming 2D diffusion.

**qPALM**
We developed a protocol for determining the channel numbers in live *E. coli* cells that allows for accurate quantification even at low levels of expression. To obtain reliable copy numbers we included (i) the maturation time, (ii) a correction for over counting due to grouped traces, (iii) blinking behavior of the fluorescent protein, and (iv) a correction for the fact that only a fraction of the cell are analyzed, because of the limiting depth of field. A detailed description is given in Supplementary Methods.

**Results**

**Functional expression of fluorescent protein-labeled MS channels**
Initially, pTRC-based vectors were created to express MscS, MscL and MscK with a C-terminal mEos3.2 and a His-tag from the *trc* promoter (IPTG inducible). We determined the activity of wild type and labeled channels in *E. coli* cells by measuring viability after a 0.3 M NaCl downshock. MscS and MscL fusion proteins increased survival of *E. coli* MJF641, a strain that lacks all seven MS channels. The protection afforded by mEos3.2-tagged channels was
slightly decreased relative to wild type channels (Supplementary Figure S1), which likely reflects differences in expression levels as indicated by Western blots. We therefore performed patch clamp analysis to record the activity of individual channels. We found measurable activities for both MscL-mEos3.2 and MscS-mEos3.2, and the conductance and relative gating tensions of the tagged channels were similar to those of the parental proteins [32] (Figure 1).

**Figure 1**: Patch clamp analysis of mEos3.2 tagged channels, benchmarked against the wild type proteins. Protoplasts prepared from (A) pBAD-MscS-mEos3.2 plasmid transformed into *E. coli* MJF429 (ΔmscS and ΔmscK) and (B) pBAD-MscL-mEos3.2 in *E. coli* MJF453 (ΔmscL and ΔmscK). The arrows indicate the opening of the channels represented. (C) Channel activity determined by patch clamp electrophysiology. The pressure ratios were determined from patch clamp measurements in giant protoplasts made from *E. coli* MJF429 (ΔmscS, ΔmscK, mscL on chromosome) that had been induced with 1 mM IPTG for 15 min to express pTRC-MscS or 0.5% L-arabinose for 65 min to express pBAD-MscS-mEos3.2; this yielded the $P_{\text{MscL-WT}} : P_{\text{MscS-WT}}$ ratio. We used *E. coli* MJF453 (ΔmscL, ΔmscK, mscS on chromosome) that had been induced with 0.5% L-arabinose for 65 min to express pBAD-MscL-mEos3.2; this yielded the $P_{\text{MscL-mEos3.2}} : P_{\text{MscS-WT}}$ ratio. The pressure ratios are given as mean ± standard deviation, and n represents the number of patches obtained to determine the pressure ratios.

MscS, MscL and MscK are evenly distributed over the membrane
We used PALM (see Text Box I) to reconstruct images of live *E. coli* MG1655 (non-fixed) in the exponential phase of growth and expressing MscS, MscL or MscK fusions with mEos3.2. In this strain chromosomally expressed MS subunits can mix and co-assemble with the respective plasmid-encoded mEos3.2 variants, creating channel complexes with different stoichiometries of non-tagged and mEos3.2-tagged proteins. With basal expression (no inducer) we observed random distribution of individual MS channels throughout the
membrane (Figure 2A; Supplementary Figure S2A and S3A). We did not observe a preference for pole localization for any of the MS channels tested. Similar data were obtained when cells were induced with 0.1 mM IPTG for 30 min (Figure 2A; Supplementary Figure S2B and S3B), except that cells expressing high levels of MscL-mEos3.2 showed spots of higher intensity along the membrane, which we attribute to clustering of MscL channels.

![Figure 2](image_url)

**Figure 2**: Distribution of fluorescently labeled mechanosensitive channels observed by PALM. (A) Membrane localization of MscS-mEos3.2, MscL-mEos3.2 and MscK-mEos3.2 in *E. coli* MG1655 without induction and with 0.1 mM IPTG (30 min induction). (B) MSD plots of trajectories determined by single-molecule tracking. Panels (B) correspond to the conditions shown in panel (A). On average about 10,000 trajectories were analyzed. Error bars indicate standard deviation.

**Table 4**: Diffusion coefficients $D$ for MscS-mEos3.2, MscL-mEos3.2 and MscK-mEos3.2 channels in *E. coli* MG1655, using leaky expression (-IPTG) and with 0.1 mM inducer (+IPTG). LacY-mEos3.2 was induced from the *rha* promoter in pACYC.

<table>
<thead>
<tr>
<th>Condition</th>
<th>MscS -IPTG</th>
<th>MscL -IPTG</th>
<th>MscK -IPTG</th>
<th>MscS +IPTG</th>
<th>MscL +IPTG</th>
<th>MscK +IPTG</th>
<th>LacY</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (*10^{-2}$ µm$^2$/s)</td>
<td>2.81</td>
<td>5.80</td>
<td>2.09</td>
<td>2.67</td>
<td>0.62</td>
<td>1.72</td>
<td>8.10</td>
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</table>
**Text Box I: Single-molecule localization microscopy**

Conventional fluorescence microscopy suffers from the drawback that the resolution at which molecules are observed is limited by the diffraction of light. In recent years single-molecule localization microscopy (SMLM) techniques have been developed, which provide images with a spatial resolution well beyond the diffraction limit. Here, we introduce two of the methods: stochastic optical reconstruction microscopy (STROM) [33] and photoactivated localization microscopy (PALM) [34, 35], which rely on the precise localization of individual fluorescent molecules.

Under the microscope a single light-emitting fluorophore yields a blurred (diffracted) spot of finite-size, which represents a cross section of the three-dimensional point spread function (PSF) (see Figure IA). The intensity profile of the blurry spot can be fitted in two dimensions with a Gaussian distribution (Figure IB). From the center point of the Gaussian fit the fluorophore is localized with high accuracy (Figure IC). According to Abbe’s criterion, the resolution limit is defined by:

\[
d = \frac{\lambda}{2NA}
\]

where \(d\) is the minimal distance between two light-emitting fluorophores can be distinguished, \(\lambda\) the wavelength and \(NA\) the numerical aperture of the microscope. Using visible light, the resolution limit \(d\) is around 250 nm. When labeling biological structures the density of the fluorophores is typically high, and the point spread functions of two adjacent fluorescent molecules are overlapping. In order to achieve super resolution images, PALM and STORM require special fluorescent probes that can be stochastically photoactivated, photoswitched, or photoconverted, which allows imaging of a subset of spatially well-separated probes at a time. Typically, many cycles of photoactivation and successive photobleaching are performed until the majority of fluorophores within the sample are localized. Finally, the obtained localizations are combined to reconstruct a super resolution image (Figure ID).

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**Figure I:** Single-molecule localization procedure. (A) Point spread function of a light emitting single point that is represented as a blurry spot in two dimensions. (B) Gaussian fit of the PSF (C) The localization of the light emitter is determined from the center points of the Gaussian fit. (D) Example of a PALM reconstruction. Magenta: nucleoid labeled with StpA-eYFP and green: cytoplasmic membrane labeled with LacY-mEos2. Image adapted from http://zeiss-campus.magnet.fsu.edu/articles/superresolution/palm/introduction.html.
For STORM small synthetic dyes are used to label subcellular targets, whereas PALM makes use of genetically-encoded fluorescent proteins. The localization accuracy is strongly dependent on the number of photons emitted. Under ideal conditions, the positions can be determined with an accuracy of 25 nm, which is roughly an order of magnitude improvement compared to conventional fluorescence microscopy.

**Text Box II: Single-molecule tracking**

With single-molecule tracking (SMT) individual molecules are traced over time. The positions of a fluorescent particle in a series of time steps, obtained from single-molecule localization microscopy, are connected to form a trajectory. The lateral diffusion coefficient $D$ is related to the mean square displacement $r^2(t)$ by:

$$r^2(t) = d2Dt^d$$

with $d$ being the dimensionality ($d = 2$ for 2D diffusion), $t$ is time, and $\alpha$ the anomalous diffusion coefficient. Assuming Brownian motion ($\alpha = 1$) the MSD increases linearly as a function of time (see Figure II). If $\alpha < 1$ the motion is called subdiffusion and the molecule’s mobility decreases with time, whereas with $\alpha > 1$ the molecule occasionally makes very long steps (superdiffusion).

SMT allows analysis of the diffusive behavior of fluorescent proteins in live cells as long as a few requirements are fulfilled. The tracer molecule must be fluorescent for a number of subsequent time steps. Ideally only one single fluorescent protein is activated at a time to avoid interlinking different molecules to one trajectory. Additionally, the displacement between two subsequent frames should be small. Shorter frame length increases the sampling rate, but simultaneously reduces the amount of detected photons per frame. SMT is therefore especially suitable for slow diffusing (membrane) proteins.

**Figure II:** Mean square displacement plots for molecules with different diffusive behavior. The MSD increases linearly for freely diffusing molecules (Brownian motion, black curve). The diffusion coefficient can be deducted from the slope. Anomalous diffusion has a non-linear relation with time (super- or subdiffusion, green and orange curve, respectively). Confined diffusion occurs when a molecule diffuses in a finite domain (blue curve).
Cluster formation coincides with reduced mobility of MscL
To analyze the dynamic behavior of the channels we performed single-molecule tracking (SMT, see Text Box II) of the mEos3.2-tagged proteins. This allowed us to track individual molecules for several time steps and to determine the lateral diffusion coefficients. The mean square displacement (MSD) of a membrane protein relates to the lateral diffusion coefficient $D$ according to $\text{MSD}(t) = 2dDt$, with $d$ being the dimensionality ($d = 2$). For the samples shown in Figure 2A, the MSD was plotted (Figure 2B) and the diffusion coefficients are shown in Table 4. MscL, MscS and MscK complexes have different masses and (predicted) radii of the membrane-embedded domains, which lead to different mobilities. The observed diffusion coefficients follow the radii of the complexes with MscL being fastest and MscK slowest. Increased expression did not significantly affect the measured mobility of MscS and MscK. In contrast, induction of MscL-mEos3.2 resulted in a roughly 10-fold reduced mobility (Table 4), which coincided with the cluster formation observed by PALM (Figure 2A, Supplementary Figure S3B). The fluorescence signal obtained from MscL-mEos3.2 upon induction originated both from the bright spots (large clusters of channels) and also from smaller clusters or single channels.

A cumulative distribution function shows heterogeneous diffusion of MscL
The degree of heterogeneity of MscL-mEos3.2 super-complexes in the membrane was investigated using a cumulative distribution function (CDF). For individual step sizes within a fixed time interval of 31 ms, obtained from single-molecule tracking, the CDF was plotted as $1 - \text{CDF}(r^2, t)$ versus $r^2$. As described in Oswald et al. the CDF of a homogeneously diffusing protein is characterized by exponential decay [36], as simulated in Figure 3A for a single population of freely diffusing membrane proteins (note the logarithmic y-axis). Two hypothetical populations at a ratio of 1:5 and diffusing with $D_1 = 0.06 \mu m^2/s$ and $D_2 = 0.008 \mu m^2/s$ show a kink in the distribution. The decay of the CDF of channels in E. coli MG1655 (IPTG induction) with mixed subunits of wild type and mEos3.2-tagged MscL is not simply biphasic (Figure 3B), suggesting that multiple populations with different mobilities are present. In contrast, in non-induced E. coli MG1655 MscL diffuses more homogeneously.

In E. coli strain MJF641, in which all MscL subunits have mEos3.2 attached, the CDF shows behavior similar to the induced state in MG1655, independent of whether the expression of the complexes was induced with IPTG or not (Figure 3C). No significant difference in MscL abundance was observed
between *E. coli* MJF641 and MG1655 as host (Figure 3D). As a control for diffusion of a monomeric membrane protein, we expressed LacY-mEos3.2 from the *rha* promoter [0.5% (w/v) L-rhamnose] at levels similar to those of IPTG-induced MscL-mEos3.2. We plotted the CDF for LacY-mEos3.2 in Figure 3B and c, which shows no visible cluster formation when expressed at similar levels as MscL-mEos3.2 (Figure 3D). The diffusion of LacY-mEos3.2 is somewhat faster than that of MscL-mEos3.2 (Table 4), but the shape of the CDF of LacY-mEos3.2 is comparable to that of MscL-mEos3.2 in MG1655 (under non-inducing conditions; Figure 3B), indicating that both are organized as monomeric membrane proteins. The deviation from a single exponential is likely caused by the fact that 3-dimensional diffusion trajectories are projected in two dimensions (on the focal plane), which distorts the distribution of step sizes [36]. Moreover, transient (weak) interactions between the proteins and

Figure 3: Cumulative distribution function (CDF) plotted as 1-P_CDF (A) of a simulated membrane protein with either a single population (open circles) or two populations (closed circles) with D₁ = 0.06 µm²/s and D₂ = 0.008 µm²/s and a hypothetical ratio of D₁/D₂ = 1:5. (B) CDF of MscL-mEos3.2 expressed in *E. coli* MG1655 with 0.1 mM IPTG (closed circles) and without IPTG (open circles). (C) CDF of MscL-mEos3.2 in *E. coli* MJF641 with (closed squares) and without (open squares) IPTG. In panel b and c the monomeric membrane protein fusion LacY-mEos3.2 expressed in MG1655 with 0.5% (w/v) L-rhamnose (triangles) was plotted as a control. (D) Average protein copy numbers per cell for the samples shown in panels B and C determined by qPALM. Numbers represent fluorescent subunits rather than fully assembled channels, and the error bars indicate the standard deviation of ~15 cells used for quantification. (E) CDF of MscS-mEos3.2 expressed in *E. coli* MG1655 with (closed circles) and without (open circles) IPTG.
components in the crowded membrane will cause anomalous diffusion, which is reflected in the CDF as well [37]. The MscS-mEos3.2 diffusion was independent from inducer concentrations in *E. coli* MG1655 (Figure 3E) and the shape of the CDF indicates no cluster formation.

**Channel copy number and confinement**

To ascertain whether the self-association of MscL is a consequence of channel abundance and reflects the presence of the mEos3.2 fluorophore, we designed pBAD vectors to express the channels from the *ara* promoter (L-arabinose inducible), which is less leaky than the *trc* promoter. Hence, we could vary the expression levels over a much wider range, i.e. from a few to 65 (MscS) or several hundreds (MscL) of channels per cell. We also analyzed the MSD of MscS and MscL in *E. coli* MJF641 when induced from pBAD (Figure 4A and B, respectively), using a range of L-arabinose concentrations (0-1%). The mobility of MscS in MJF641 was independent of channel abundance but somewhat slower than in *E. coli* MG1655, which is possibly due to the larger drag on the channel because more fluorescent proteins are attached. The slope of the MSD of MscL decreased with increasing channel number per cell, which is indicative of clustering. If we assume a distribution of cluster sizes then the immobile fraction becomes more prominent as channel numbers increase. These data also suggest that mEos3.2 predisposes the MscL channel to form clusters. This may arise through collisions of pre-formed complexes rather than at the folding or assembly stage as fluorescent β-barrel proteins typically mature (fold) more slowly than other proteins. In contrast, for MscS-mEos3.2 the spatial separation of the seven mEos3.2 fluorophores is sufficiently great that they

![Figure 4](image)

*Figure 4:* MSD plot of trajectories of mechanosensitive channels expressed in *E. coli* MJF641 (A) MscS-mEos3.2 and (B) MscL-mEos3.2 expressed from the *ara* promoter in pBAD. The numbers represent the average channel number per cell ± standard deviation, obtained from independent experiments by varying the inducer concentration from 0 to 0.5% L-arabinose. 200-2000 trajectories were analyzed and error bars represent standard error of the mean (SEM).
cannot interact to form clusters. In this context, it is possibly significant that MscS can tolerate additions to the carboxy-terminus (e.g. the alkaline phosphatase protein) and the 18 residues at the carboxy-terminus can be deleted without significantly impacting folding, assembly or function [20].

**Survival versus channel number**

An outstanding question is the number of channels that are required to confer protection from an osmotic downshock. We used *E. coli* MJF641 as the host strain and grew the cells in high-salt Luria Broth (LB containing 0.3 M NaCl; 0.78 ± 0.01 Osm), and varied the amounts of either MscS-mEos3.2 or MscL-mEos3.2 using the *ara* promoter system (*L*-arabinose at 0 to 1% w/v). MscK was not considered for this experiment, since its contribution to osmoprotection from a fast downshock is negligible. After the induction period of 1 h, a fast 0.3 M NaCl downshock was applied as described in Materials and Methods. At the same time the remaining non-shocked cells were washed twice in high-salt PBS (PBS adjusted to 0.78 ± 0.01 Osm with NaCl) and resuspended in equal volumes of the same buffer. Cells were kept in high-salt PBS for 5 h at 37 °C prior to quantification with qPALM to ensure complete maturation of all mEos3.2 proteins (maturation half time $t_{1/2} = 40-45$ min, see Supplementary Figure S7). A detailed description of how channel numbers were quantified can be found in the Supplementary Methods. The collective survivability-qPALM data is shown in Figures 5A and B. For MscS and MscL channels, the

![Figure 5](image-url)

**Figure 5:** Survival after osmotic downshock of *E. coli* MJF641 as a function of channel copy number for (A) MscS-mEos3.2 or (B) MscL-mEos3.2. The osmotic downshock was applied by rapid mixing of cells adapted to LB plus 0.3 M NaCl into LB. The channel copy number was varied with different inducer concentrations (0 to 1% w/v *L*-arabinose). The average cell survival was determined by colony counting of shocked and non-shocked cells. The channel numbers were quantified after cells were incubated for 5 h in PBS to ensure complete maturation of mEos3.2. For each condition between 6 and 25 cells were analyzed by qPALM and the median of different assays with various inducer concentrations is plotted individually. Error bars represent standard deviation of quantified channel numbers.
survivability after the rapid 0.3 M NaCl downshock increased almost linearly with the number of channels. Around 60% of the cells expressing MscS-mEos3.2 survived the downshock when 55 channels per cell were present. We could not increase the MscS channel number beyond this average, which may explain why a fraction of cells did not survive the osmotic downshock. With MscL-mEos3.2 we reached 100% survival with an average of 100 or more channels per cell.

To ascertain that *E. coli* is still viable and the MS channels are functional after the incubation of the cells in high-salt PBS, we adapted *E. coli* MJF641 to high-salt LB and expressed either MscS-mEos3.2 or MscL-mEos3.2 from the *ara* promoter (0.5% L-arabinose (w/v) for 1 h). We then performed the osmotic downshock immediately after the induction period and after 5 h of incubation in high-salt PBS. Supplementary Figure S6A shows that the overall number of cells only slightly decreased after 5 h in high-salt PBS and the survival of cells before and after 5 h incubation in high-salt PBS was comparable (Supplementary Figure S6B).

**Discussion**

In bacteria MS channels play a key role in osmoregulation. MscS and MscL are the principal channels required for cells to survive hypoosmotic transitions. Other MS channels contribute at their usual levels of expression to varying extents but the ultimate outcome, cell survival, is dependent on complex parameters, including the rate of the shock [12], specific channel gating properties, the overall number of MS channels and perhaps other as yet unidentified factors. Here, we provide a quantitative analysis of the contribution of MscS and MscL to osmoprotection and provide insights into their *in vivo* functioning. We determined channel abundance, aggregation state, localization in the membrane, and lateral diffusion. Despite the lack of organelles, it is increasingly clear that bacteria have well-organized compartments. Not only does the cytoplasm show confined structures, e.g. the nucleoid or separated areas of increased activity for transcription and translation [38, 39], but also the membrane seems to be more structured than initially expected for prokaryotic forms of life. The concept of bacterial lipid domains is under discussion, e.g. in *B. subtilis* processes such as peptidoglycan synthesis have been proposed to increase the local complexity of the cytoplasmic membrane [40]. The chemotaxis network in *E. coli* was shown to form clusters mainly at the cell poles, which might be a mechanism to increase
their sensitivity by amplifying external signals from neighboring receptors [4, 41]. For mechanosensitive channels protein-lipid interactions form the basis for their gating and they are affected by membrane curvature. Thus knowledge about the localization and organization of the proteins in the membrane is crucial. We determined the localization of MscS, MscL and MscK in the *E. coli* plasma membrane with PALM; super-resolution optical microscopy has to the best of our knowledge not been used to localize MS channels. Reconstructions of cells expressing fluorescently labeled MscS, MscL and MscK showed an equal membrane staining. In a previous study, using FlAsH-tagged MscL and MscS, a small fraction of the channels localized to the poles of the cells [27], however our results suggest that all channels tested have no preference for the cell poles.

The Saffman-Delbrück relationship for free diffusion in biological membranes predicts that the diffusion coefficient scales logarithmically with the hydrodynamic radius of the transmembrane domain [42], which has been verified experimentally in proteoliposomes [43]. On the basis of the available crystal structures, the radii of the membrane-embedded domain of MscL and MscS are ~2.5 and 4 nm, respectively. There is no structural information of MscK but the protein is much larger (7x11 transmembrane segments, TMS) than MscS (7x3 TMS) and may have a radius of 7-8 nm. The here determined lateral diffusion coefficients suggest a stronger dependence on protein radius than predicted by the Saffman-Delbrück model. We note that the cytoplasm and periplasm are highly crowded and more viscous than water [44, 45]. Since MscS has large cytoplasmic domains and MscK has large cytoplasmic and periplasmic domains, these proteins will experience more drag than MscL, which is not accounted for in the Saffman-Delbrück model. The retardation will be even more pronounced when one or more subunits are tagged with a fluorescent protein..

When MscL-mEos3.2 was expressed in *E. coli* MJF641, where each MscL subunit is tagged, channel clustering was observed even at the lowest levels of expression (Supplementary Figure S4). This uneven distribution of channels is not what one would expect for Brownian diffusion of individual particles as shown by simulations (Supplementary Figure S5). If only one or a few of the subunits of MscL were tagged with mEos3.2, the channels exhibited a high mobility and a more even distribution (Figure 2). At higher levels of expression of tagged proteins, clusters were formed even in the presence of wild type polypeptides. We attribute the clustering of MscL as labeling artifacts that appear when the majority or all of the subunits of the pentameric channels are
tagged with mEos3.2. Our single-molecule tracking experiments are entirely consistent with the observation of clusters in PALM reconstructions. We find that the diffusion of MscL (clustered in *E. coli* MJF641) is anomalous with signs of confinement at longer timescales, especially with increasing channel copy numbers (Figure 4B). The cumulative distribution function provided quantitative information on the cluster formation that cannot be deduced from the mean square displacement analysis. Where clustering was observed the CDF showed multiple populations. We were not able to fit the data with a multicomponent linear equation since the graph shows a smooth transition from slowly diffusing clusters to faster, smaller aggregates and individual channels. We used LacY-mEos3.2, a well-characterized, monomeric membrane protein, as a control and observed that the shape of the CDF of MscL-mEos3.2 (at low inducer concentration) and LacY-mEos3.2, expressed in *E. coli* MG1655 point towards a similar diffusional behavior with no signs of protein clustering.

In theory, cluster formation can occur when channels have a minimum of two fluorescent tags weakly interacting with each other. Different scenarios of expressing MscL-mEos3.2 in *E. coli* MG1655 or MJF641, are depicted in Figure 6A and B. Remarkably, under conditions where we find (predominantly) clusters, the channels protected *E. coli* against hypoosmotic stress and the conductance and gating properties were similar to those of (non-clustered) wild type MscL. In our studies, individual channels are tied together via the cytoplasmic mEos3.2 label and not by the tension-receiving transmembrane

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**Figure 6:** Cluster formation of MscL depends on the number of mEos3.2-labelled subunits in the channel. (A) In *E. coli* MG1655, using mild expression conditions, the plasmid-derived MscL-mEos3.2 mixes with chromosome-derived MscL subunits. In most cases only one or two subunits are labeled. (B) In *E. coli* MJF641, all subunits are labeled, which reduces rotational freedom of the fluorescent protein and leads to artificial cluster formation of MscL channels. Cluster formation can also occur when MscL-mEos3.2 is overexpressed in *E. coli* MG1655, that is, when the majority of subunits have a fluorescent protein moiety.
surface, which apparently does not affect the gating activity. It should be noted though that other studies have observed MscL clustering in *E. coli* [46, 47] as well. In these studies MscL was labeled with GFP, which has a much higher tendency for self-association than mEos3.2. Grage et al. observed clustering of an Alexa-488 labeled MscL reconstituted in lipid vesicles by small-angle neutron scattering (SANS), atomic force microscopy (AFM), and fluorescence microscopy [28]. Their continuum mechanics simulations shows that organizing MscL channels in clusters minimizes their energy and lowers their activity. In brief, they argue that channel opening increases their surface area and clustering of the proteins would thus hamper their gating. Thus clustering of MscL could be an activity regulation mechanism, e.g. by membrane-mediated (anti-) cooperative gating [48, 49], however our data indicate that native channels do not cluster.

We provide evidence that genetic labeling of a membrane protein like MscL can give rise to artifacts, in particular when proteins are overexpressed. Fluorescent proteins can cause mislocalization when fused to homooligomers [50] and have the tendency to self-associate. Even though monomeric variants like mEos3.2 have been engineered with a *K*ₐ for self-association > 480 µM [51], when linked to a membrane protein artificial clustering may still occur. For membrane proteins one needs to take into account the net effect of local protein concentrations, orientational restriction, and volume exclusion [52]. The largest contribution to the enhancement of self-association of proteins in membranes originates from the restriction in translational mobility, which manifests itself as an increased local concentration. Grasberger et al. estimated that for a cylindrical-shaped membrane protein its propensity to form dimers can be 10⁶-times higher than for isotropic diffusion of water-soluble proteins [52]. This situation may not hold for MscL-mEos3.2, since the putative oligomer-forming moiety is not embedded in the membrane but rather connected to a membrane protein via a small linker. The rotational freedom of mEos3.2 is presumably higher than that of the membrane protein itself, but certainly lower compared to a cytoplasmic protein of the same size. The situation becomes more pronounced when five fluorescent moieties are situated in close proximity to each other and to the membrane, which is the case when all MscL subunits are labeled with mEos3.2 (Figure 6B). We note that MscS and MscK, with seven subunits, did not show the clustering behavior. From crystal structures it is known that MscL subunits are more closely packed to each other at the C-terminus than those of MscS. The rotational freedom of mEos3.2 in MscL may thus be much smaller than in MscS. This decrease in entropy may lead to dimerization of mEos3.2 not only within a channel but also between channels.
The clustering of channels through mEos3.2 apparently leaves sufficient freedom for MscL to undergo the large conformational changes from closed to open.

Li and co-workers used a genome-wide approach, based on ribosome profiling, to determine protein copy numbers by assuming a constant rate of protein synthesis and turnover for all bacterial proteins [53]. Their deduced copy numbers for MscS and MscL suggest that they are relatively abundant in the cell (around 600 channels of MscS and MscL in cells growing in rich medium). Using Western blot analysis and fluorescence microscopy, MscL was detected in the range of 300-1000 channels per cell [46]. Electrophysiology studies showed lower copy numbers ranging from 20-40 MscS and 5-40 MscL channels per cell [54, 55], which most likely reflects the fact that not all channels gate in patch clamp experiments, whereas Western blotting or ribosome profiling detects the entire amount. With the multiplicity of MS channels in *E. coli* and deduced protein numbers, the question arose, how many channels are required to confer protection from a given downshock.

To address the relationship between cell survival after hypoosmotic shock and channel abundance, we performed viability experiments based on colony counting, using an *E. coli* strain lacking all seven native MS channels (MJF641) and expressing various amounts of plasmid-derived MscS-mEos3.2 or MscL-mEos3.2. We observe a variation in survivability of 15% or higher, which is not unusual for colony counting in plating experiments [56]. Alternatively, survivability can be determined with flow cytometry [57] or by time-lapse imaging in microfluidic flow cells [12]. We developed a reliable protocol of protein quantification by qPALM, which allowed us to correlate the cell survival with channel copy numbers for each expression condition. Our qPALM approach is based on the work of Annibale et al. [58] but tailored for *in vivo* application with diffusing fluorescent probes. Quantitative microscopy faces several challenges, which can lead to over- or underestimation of the protein copy numbers. Detailed knowledge about the photo-physical properties of the fluorescent protein allows correcting for photoblinking and maturation. However, the photo efficiency of photoactivatable proteins poses some uncertainty that we could not correct for. The determined copy numbers therefore represent a lower bound and might be somewhat higher (see Supplementary Methods and Supplementary Figures S7-S9 for more information).

We found that the channel numbers correlate more or less linearly with survival after a 0.3 M NaCl downshock. Approximately 100 MscL-mEos3.2 channels are required for full protection (see Figure 5). We did not succeed in
expressing MscS-mEos3.2 to similarly high levels and thus did not reach full protection, but by extrapolation the same number may apply for MscS. The highest MscL channel number we achieved by induction with L-arabinose was on average 400 per cell, which is close to native expression based on ribose profiling and immunoblotting [46, 53]. It was recently shown that the speed at which the cells experience the downshock is an important determinant for cell survival [12]. The chances of cell survival significantly increased if the downshock was applied over a longer period, and with slow enough downshock even MscK protected the cell from lysis. Thus, our correlation between cell survival and channel numbers shown in Figure 5 is valid for a fast 0.3 M NaCl downshock applied by rapid pipetting. Booth calculated that upon a 0.5 M downshock five MscS channels would in theory suffice to deplete the cell of its ion pool within 200 ms [59]. Our results suggest that the flux through the channel is lower or that gating takes longer than is typically assumed. One reason might be that during an osmotic downshock the membrane potential collapses and consequently the flux of solutes through the channels is substantially reduced.

In summary, we show for MscL that above 100 channels all cells survive the imposed osmotic stress and that a similar quantitative relationship is likely for MscS. Additional channels might be needed when cells face a downshock of higher magnitude [19]. We show that MscS, MscL and MscK are homogenously distributed over the membrane with no indications for clustering of the channels themselves. The diffusional behavior of MscL and MscS is that of free channels with some indications for confinement on longer timescales, which may reflect heterogeneity in macromolecular crowding or local differences in lipid composition of the membrane.

References


Supplementary information:

On the mobility, membrane location and functionality of mechanosensitive channels in *Escherichia coli*

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Supplementary Methods
Supplementary Figures: Fig. S1-S9
Supplementary Methods

Quantification of MS channels fused to mEos3.2 using qPALM

Quantitative photo-activated localization microscopy (qPALM) of fluorescently labelled membrane proteins requires detailed knowledge about the photophysics of the fluorescent protein. The fluorescent protein mEos3.2 was chosen, because of its superior properties in term of brightness and monomeric character [1]. Photo switching was well controlled by varying the power of the activating laser (405 nm), making it ideal for quantitative microscopy. We developed a protocol for determining the channel numbers in live E. coli cells that allows quantification even under low expression conditions. To obtain reliable copy numbers we included in the analysis: (i) the maturation time, (ii) a correction for over counting due to grouped traces, (iii) blinking behavior of the fluorescent protein, and (iv) a correction for the fact that only a fraction of the cell can be seen, because the depth of field is limited.

(i) Maturation time

We determined the *in vivo* maturation time of the fluorescent protein by expressing soluble mEos3.2 protein in *E. coli* MC1061 under anaerobic growth conditions at 37 °C. After addition of 32 µg/mL chloramphenicol the culture was aerated and the emerging fluorescence was measured using flow cytometry, as shown in Fig. S7. We observed maturation of mEos3.2 with two distinct kinetic phases. The maturation half times ($t_{1/2}$) were obtained by fitting the data points to a multi-exponential function. We find $t_{1/2}$ values for mEos3.2 maturation in live *E. coli* cells at 37 °C of <5 min for the first phase and 40-50 min for the second phase. Cells used for channel counting were kept for 5 h at isosmotic PBS at 37 °C in all cases to assure full maturation (avoiding undercounting due to incomplete maturation).

(ii) Single molecules as grouped fluorescent traces

One convenient fact when quantifying membrane proteins is their slow diffusion. Membrane proteins hardly change their position in between two frames. Proteins that are fluorescent for several consecutive frames can therefore be grouped together and counted as one molecule. We immobilized purified mEos3.2 on a clean cover slide and observed its fluorescent properties using the same laser settings as for a live sample. The counts obtained from PALM were scanned for grouped traces using a custom written ImageJ macro. The algorithm finds traces by grouping counts that occur within a radius of 4 pixels for a series of frames. The distribution of the fluorescence ON time ($t_{on}$) was plotted as number of frames in Fig. S8a. Counts that were only detected in one frame and cannot be grouped are represented in magenta. Traces longer
than one frame can be fitted with an exponential \( y = a \cdot \exp(b \cdot x) \); the obtained values were \( b = -0.06778 \) and \( a = 29,128 \). The resulting fit reflects the \( t_{on} \) of long-lived molecules very well, but the measured counts for short-lived molecules with a \( t_{on} \) of 1 frame or less are higher than predicted from fitting and extrapolating to \( x = 1 \). We assume that the \( t_{on} \) of fluorescent proteins follows a single exponential decrease and determine the number of mEos3.2 molecules as defined by the theoretical, extrapolated fit (blue bar Fig. S8a). The counts represented by the magenta bar can be attributed to background and auto fluorescence, which is wrongly detected by the algorithm. Like that the half-time of the fluorescence \( t_{on\ 1/2} \) of mEos3.2 was calculated to be 30.7 ms. For all live samples we used the same fit to estimate the number of ungrouped molecules. Fig. S8c-e show the distribution of ON times for three expression levels induced with 1%, 0.25% and 0.01% in descending order. Finally, it is essential that all fluorescent proteins present in the sample are activated within the time of acquisition. The acquisition needs to last at least until the time when the total detected counts per frame reach zero, as can be seen in Fig. S8b.

(iii) Blinking

mEos3.2 can exist in three different states: activated, dark and bleached. Many fluorescent proteins can blink, which means they can repeatedly switch between activated and dark states, before they bleach. This leads to overestimation of protein copy numbers when grouping fluorescent traces. We corrected for that by introducing a dark time \( t_d \) in our analysis, in which the protein is dark, but not yet bleached and can reoccur after several frames. This additional parameter must be chosen carefully when grouping fluorescent traces because it defines how long a fluorescent protein is allowed to stay in a dark state in order to be counted as one grouped trace. When the dark time \( t_d \) is not long enough one fluorescent protein might be interpreted as more than one molecule. In the work of Annibale et al. the photoblinking behaviour of mEos2 was analysed [2]. We adapted their strategy and tailored it for our purpose. They observed that with longer \( t_d \) the counts of immobilized mEos2 molecules reach a more accurate number. They found that the decreasing curve could be fitted with the following semi-empirical equation:

\[
N(d_t) = N \cdot \left(1 + n_{\text{blink}} \cdot e^{\frac{1-d_t}{t_{\text{off}}}}\right)
\]

where \( N \) represents the actual number of fluorescent proteins in the sample, \( n_{\text{blink}} \) is the number of blinks and \( t_{\text{off}} \) the dark time. However, there is a downside when quantifying proteins in live cells, which has to do with their
diffusion during the image acquisition. This makes qPALM on live cells challenging, because even membrane proteins can diffuse long distances during image recording of several minutes. In the previous section the method for determining grouped traces is described. To correct for the fact that the detected counts do not allow any dark time \((t_d = 0)\), we determined the blinking behaviour of mEos3.2 immobilized on cover slides as described before. In Fig. S9 we plot the counts per \(\mu m^2\) as a function of dark time \(t_d\). The ratio of counts obtained from fitting the curve with the semi empirical equation and the counts detected without dark time is \(\gamma = \frac{N}{N_{gt}} = 0.697\), which is the correction factor for fluorophore blinking. Using this approach we reduce the risk of overcounting due to blinking. It should be mentioned that this attempt fails to give accurate numbers when not all mEos3.2 proteins are activated. In Ulrich et al. they find about 20% of GFP-type fluorescent proteins stay in the dark and will thus not be detected [3]. Because photo efficiency is dependent on many factors, like pre-treatment of the sample, type of organism and laser intensity for activation and readout, we decided not to correct for photo efficiency. We likely underestimate the copy numbers for MS channels by about 20%. The actual channel numbers will thus be somewhat higher depending on the photo efficiency of mEos3.2.

(iv) Depth of field
We determined the depth of field to estimate what fraction of the cell is out of the observable area. We performed a z-stack with epi-illumination over a range of 4 \(\mu m\) with increments of 0.02 \(\mu m\) on immobilized fluorescent beads and determined the upper and lower limit at which the peak fitter can still detect the fluorophores. The laser power was decreased to match the intensity profile of the fluorescent beads to the one from mEos3.2 molecules. The depth of field was determined to be \(d_z = 0.78 \mu m\). With a width of 1 \(\mu m\) an E. coli cell would almost fit completely in the depth of field, except the upper and lower part of the cell’s cylinder including the membrane. Due to the relatively big depth of field, we decided not to introduce a correction for the limiting depth of field, because proteins are very likely to diffuse to the observable area of the membrane within the long acquisition time.
Supplementary Figures

**Fig. S1.** Survival of *E. coli* MJF641 cells transformed with MscS or MscL with and without mEos3.2 tag. Samples were induced with 0.3 mM IPTG for 15 min prior to being subjected to a 0.3 M NaCl downshock. As a control *E. coli* MJF641 without plasmid was subjected to the same osmotic downshock. Error bars represent standard deviation.
Fig. S2. PALM reconstructions of *E. coli* MG1655 harbouring pTRC-MscS-mEos3.2 with (A) leaky expression and (B) induction with 0.1 mM IPTG for 30 min. Scale bar: 2µm.
Fig. S3. PALM reconstructions of *E. coli* MG1655 harbouring pTRC-MscL-mEos3.2 with (A) leaky expression and (B) with 0.1 mM IPTG for 30 min. Cluster formation is visible in cells that were induced. Scale bar: 2 µm.
Fig. S4. qPALM of *E. coli* MJF641 expressing MscL-mEos3.2 from pBAD plasmid at various L-arabinose concentrations. Cells were grown in LB medium at 37 °C and, after induction, suspended in isosmotic PBS to allow completion of mEos3.2 maturation for 5 h. Qualitatively similar images and clustering of MscL-mEos3.2 were obtained after 1h of induction, that is after the majority of the protein had matured (see Fig. S7). Scale bar: 2 µm.

Fig. S5. Simulated distribution of (A) 100 or (B) 400 particles freely diffusing along the cell membrane of a cell with length of 3 µm and a width of 0.8 µm and a hypothetical diffusion coefficient of 0.06 µm²/s. The Smoldyn software (http://www.smoldyn.org) was used to simulate Brownian diffusion of particles for 100 s. We depict the distribution of the particles along the membrane in a field of view of 0.5 µm, which is similar to the presentation of the qPALM data.
**Fig. S6.** Cell survival in LB and PBS. *E. coli* MJF641 adapted to high-salt LB and expressing either MscL-mEos3.2 or MscS-mEos3.2 for 1 h with 0.5 % L-arabinose. (A) Cell counts per mL by colony counting on LB-agar plates directly after the induction period or after 5 h incubation in high-salt PBS. (B) Cell survival from a rapid 0.3 M NaCl downshock immediately after induction (indicated by high-salt LB) and after 5 h incubation in high-salt PBS.

**Fig. S7.** In vivo maturation of mEos3.2. *E. coli* MC1061 expressing cytoplasmic mEos3.2 from pBAD was grown anaerobically in LB medium at 37 °C. After aeration of the culture (t = 0) the folded and non-fluorescent protein was allowed to mature. At indicated time points fluorescence was measured with flow cytometry. The fluorescence appears in two phases, one initial fast phase of maturation (and $t_{1/2} < 5$ min) and a second phase characterized by a lower rate constant ($t_{1/2}$ of 40-50 min).
**Fig. S8. In vitro and in vivo counting of mEos3.2 molecules.** (A) Purified mEos3.2 molecules were immobilized on a cleaned cover slide and the ON time ($t_{on}$) was analysed by grouping fluorescent traces. Each frame was 31 ms. Grouped traces with a length between 2 and 19 frames (red bars) were fitted with an exponential decay and extrapolated to $x = 1$ (blue bar). The blue bar + magenta bar represent the counts that were only fluorescent for one frame. To determine the actual copy number of a fluorescent protein, the grouped traces of various lengths were summed up together with the calculated counts that only last for one frame. (B) Histogram of counts detected in *E. coli* cells expressing MscL-mEos3.2 as a function of movie acquisition time (bin size 180 counts). Towards the end of the movie there were no counts anymore, indicating that all mEos3.2 molecules were activated. (C) qPALM of MscL-mEos3.2 in *E. coli* MJF641 cells, induced with 1% (D), 0.25% and (E) 0.01% of L-arabinose. Representative cells are shown in the right panels; fluorescence counts are plotted as function of ON time (number of frames). The data were fitted to a single-exponential decay function and the counts with varying ON times were summed up to obtain the counts per cell.
Fluorescent counts versus dark times $t_d$. The fluorescence of surface-immobilized mEos3.2 was measured over time (frames of 31 ms) to quantify the blinking behaviour of mEos3.2. In order not to overestimate the real number of molecules due to blinking, we corrected for the time mEos3.2 spends in the dark state (for details of method see ref. [2]). The data points can be fitted with a semi-empirical exponential function as described in Supplementary methods. The ratio of directly counted molecules and actual molecules was $\gamma = 0.679$, meaning that without correction the quantification would lead to an overestimation of around 30%.

Fig. S9.

References
