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### Single-molecule specific mislocalization of red fluorescent proteins in live Escherichia coli

Harshad Ghodke

*University of Wollongong, Rijksuniversiteit Groningen, harshad@uow.edu.au*

Victor E. A Caldas

*University of Groningen*

Christiaan M. Punter

*University of Groningen*

Antoine M. van Oijen

*University of Wollongong, vanoijen@uow.edu.au*

Andrew Robinson

*University of Wollongong, Rijksuniversiteit Groningen, andrewr@uow.edu.au*

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# Single-molecule specific mislocalization of red fluorescent proteins in live *Escherichia coli*

## Abstract

Tagging of individual proteins with genetically encoded fluorescent proteins (FPs) has been used extensively to study localization and interactions in live cells. Recent developments in single-molecule localization microscopy have enabled the dynamic visualization of individual tagged proteins inside living cells. However, tagging proteins with FPs is not without problems: formation of insoluble aggregates and inhibition of native functions of the protein are well-known issues. Previously reported artifacts manifest themselves at all expression levels of the FP-tagged proteins, making the design of control experiments relatively straightforward. Here, we describe a previously uncharacterized mislocalization artifact of *Entacmaea quadricolor* red fluorescent protein variants that is detectable at the single-molecule level in live *Escherichia coli* cells.

## Keywords

specific, proteins, mislocalization, red, live, single-molecule, fluorescent, escherichia, coli

## Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

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## Single-molecule specific mislocalization of red fluorescent proteins in live *Escherichia coli*

Harshad Ghodke,<sup>\*†</sup> Victor E. A. Caldas,<sup>\*</sup> Christiaan M. Punter,<sup>\*</sup> Antoine M. van Oijen,<sup>†</sup> and Andrew Robinson<sup>\*†</sup>

<sup>\*</sup> Zernike Institute for Advanced Materials, Rijksuniversiteit Groningen, Nijenborgh 4, 9747 AG, The Netherlands; <sup>†</sup> School of Chemistry, University of Wollongong, NSW, 2522 Australia.

**ABSTRACT** Tagging of individual proteins with genetically encoded fluorescent proteins (FPs) has been used extensively to study localization and interactions in live cells. Recent developments in single-molecule localization microscopy have enabled the dynamic visualization of individual tagged proteins inside living cells. However, tagging proteins with FPs is not without problems: formation of insoluble aggregates and inhibition of native functions of the protein are well known issues. Previously reported artifacts manifest themselves at all expression levels of the FP-tagged proteins, making the design of control experiments relatively straightforward. Here, we describe a previously uncharacterized mislocalization artefact of *Entacmaea quadricolor* red fluorescent protein (RFP) variants that is detectable at the single-molecule level in live *E. coli* cells.

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Biological imaging, in particular fluorescence microscopy, has revolutionized our understanding of many biological processes. In particular, the ability to endogenously tag proteins in cells using genetic fusions of fluorescent proteins has provided important insights into sub-cellular localizations and structures formed by proteins in live cells as part of normal metabolism (1). While genetic fusions of fluorescent proteins represent the most convenient strategy to image biological phenomena while approximating native conditions, they are not without problems. Besides well known problems of aggregation and modification of function, recent reports provide evidence for other types of artefacts related to the fusion protein, substantiating the observation that fluorescent proteins are not inert reporters. Notably, it has been reported that Clp proteins in *Escherichia coli* formed artefactual clusters when tagged with particular FPs (2). More recently, it has been demonstrated that photoswitchable FPs can influence the localization of DNA-binding proteins in *E. coli* (3).

Red fluorescent proteins constitute an important part of the fluorescent toolkit, not only because they extend the number of available channels for imaging, but also because cellular

autofluorescence tends to be lower in these channels. Dimerization of RFPs is a well known downside, but the latest generation RFPs promise to be monomeric and possess much improved photophysical properties (4, 5). To test the suitability of these RFPs for live cell imaging in *E. coli*, we expressed these RFPs in the absence of any tagged protein and uncovered a mislocalization artefact of RFPs that is detectable at the single molecule level.

We expressed the *Entacmaea quadricolor* RFP derivative, mKate2 (5) from an arabinose-inducible pBAD promoter at single-molecule levels in *E. coli* (MG1655) cells (Fig. 1A; see Supporting Material). Fluorescence (and hence, copy numbers) of mKate2 could be tuned by varying the concentration of L-arabinose in the growth medium (See Supporting Material Fig 1) (6). Unexpectedly, fluorescence images of individual cells revealed that mKate2 exhibits distinct, dynamic foci when expressed at very low levels. These foci localize at the cell periphery, suggestive of association with the membrane (Fig 1A and movie 1). It should be noted that in the emission window corresponding to the red channel, MG1655 does exhibit cellular autofluorescence (see SI Fig 2A). However, this cellular autofluorescence does not exhibit the

membrane localization observed in MG1655 expressing mKate2 (compare Supporting Material Fig 2A with Fig 1A). Further, when imaged and analyzed identically, a greater number of localizations are obtained in mKate2 strain compared to the WT background (39 vs. 8 peaks/cell for the mKate2 MG1655 strain and WT MG1655 respectively) consistent with leaky expression of mKate2 from the pBAD promoter. This localization is consistent with that of the FP-tagged membrane protein LacY-mCardinal (Fig. 1B) (4) and distinct from that of the nucleoid-associated replisome protein DnaQ-YPet (the  $\epsilon$  subunit of DNA polymerase III tagged with the YFP variant YPet; Fig. 1C). At higher concentrations of L-arabinose (0.1%), the localization of mKate2 resembles that of a cytosolic protein (Fig. 1A).

There are at least two possibilities for why membrane-associated foci may not be visible at higher mKate2 concentrations. The first is that at higher concentrations there is no longer sufficient contrast to distinguish individual membrane-bound mKate2 molecules as foci above the diffuse background of cytosolic molecules. A second possibility is that the distribution of mKate2 molecules between the membrane and cytosol changes as a function of mKate2 concentration, with a greater proportion of molecules bound to the membrane at low concentrations. In our previous work we demonstrated under similar conditions that we could detect a population of monomeric, membrane-bound UmuC-mKate2 molecules against a background of cytosolic mKate2 (7), indicating that our ability to detect foci is not limited by contrast in this concentration regime.

Expressed without fusion partners, YPet did not exhibit clear foci and appeared to be homogeneously cytosolic (Supporting Material Fig. 2B), whereas the mKate2 derivative mCardinal exhibited distinct foci at low expression levels (Supporting Material Fig. 2C). The membrane-foci artefact was not detected in MG1655 exhibiting leaky expression of mCherry under the pBAD promoter (Supporting material Fig. 2D). Together these observations suggest

that artefactual membrane localization may be specific to *E. quadricolor* TagRFP derivatives.

As the artefact is evident when imaging at very low expression levels, analyzing the localization of low copy number RFP fusions should still be possible provided that appropriate control measurements are made. For example, in our recent work on the localization of DNA polymerase V in *E. coli* we observed an interaction between the fusion protein UmuC-mKate2 and the cell inner membrane (7). To demonstrate that membrane interaction is a *bona fide* property of UmuC, as opposed to an artefact introduced by mKate2, we used an inducible plasmid to increase the concentration of mKate2 to levels where the artefact could no longer be detected and showed that foci of UmuC-mKate2 remained visible on the membrane. As an additional confirmation, electron microscopy revealed the membrane association of wildtype UmuC in fixed cells, using antibody conjugated gold nanoparticles.

These findings support previous work indicating that fluorescent proteins and their derivatives from different organisms possess characteristics that can differentially influence the behavior of the tagged protein. Control experiments should account for the possibility that some artefacts may manifest at certain expression levels. These findings underscore the importance of verifying observations of fluorescently tagged proteins with orthogonal techniques or multiple tags wherever possible.

### Supporting Material

Supporting materials and methods accompany this letter.

### Author Contributions

HG cloned the constructs used in this study. HG, VC and AR performed the microscopy experiments. HG, VC and CMP wrote the code used to analyze data with inputs from AR. AvO and AR supervised the work. Manuscript was written with input from all authors.

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acquisitions filtered to enhance peaks (third row), reconstructed images and short-axis projections (bottom) of **A.** mKate2 expressed under a pBAD promoter in the presence of varying amounts of L-arabinose [0 ( $n_{\text{cells}} = 142$ ,  $n_{\text{peaks}} = 5,575$ ),  $5 \times 10^{-3}$  ( $n_{\text{cells}} = 137$ ,  $n_{\text{peaks}} = 8,085$ ),  $10^{-1}$  ( $n_{\text{cells}} = 149$ ,  $n_{\text{peaks}} = 13,283$ ) percent **B.** mCardinal fusion of LacY expressed under a pBAD promoter on a plasmid in fixed *E. coli* MG1655 ( $n_{\text{cells}} = 26$ ,  $n_{\text{peaks}} = 22,954$ ). **C.** a chromosomally expressed YPet fusion of dnaQ ( $\epsilon$  subunit of polymerase III) in live *E. coli* MG1655 ( $n_{\text{cells}} = 154$ ,  $n_{\text{peaks}} = 277$ ). Scale bar represents 2.0  $\mu\text{m}$ . Short axis distribution histograms were normalized for number of cells measured.

## Figure legend:

Bright-field (top), average projections of fluorescence acquisitions (second row) and