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CRISPR/Cas9-mediated genome editing assists protein dynamics studies in live cells

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ABSTRACT

Spatial and temporal regulation of molecular reactions dictates cell fate. Thus, studying molecular dynamics is essential to understand how cells decide what to do and the fundamental perturbations causing disease. Classically, molecular dynamics has been studied by protocols based in the overexpression of fluorescent fusion proteins. However, overexpression is associated to altered stoichiometry, molecular dynamics and subcellular distribution. We here discuss the necessity to study molecular dynamics of fluorescent fusion proteins expressed under physiological mechanisms in the cell, pointing to CRISPR/Cas9-mediated genome editing as the ideal means to do so. Current genome editing protocols enable us to study molecular dynamics while avoiding drawbacks associated to overexpression.

Overexpression has been the principal means to study the molecular dynamics in live cells. However, cell biologists are now aware of the multiple consequences of ectopic overexpression of proteins, including the perturbation of protein localization, molecular dynamics and stoichiometry (Gibson et al., 2013). Proteins have a busy 'social life' inside cells and both the place and the frequency of meetings with their partners are tightly regulated. In this context, most proteins are continuously part of molecular complexes organised under a particular physiological stoichiometry, which is essential to achieve the proper function of such complexes and eventually the healthy response and fate of the cell.

Thus, to understand how cell behaviour is regulated, it is essential to study the dynamics of proteins by using approaches, which avoid overexpression and the collateral dramatic perturbations mentioned above. To achieve this aim, researchers ectopically express a recombinant version of the protein under study in a knock-out background with close to endogenous levels (Schluter et al., 2006). However, in an optimal scenario, we need to track under the microscope endogenous molecules, whose expression levels are regulated by physiological mechanisms. Once this is achieved, microscopy techniques with high spatial and temporal resolution are needed to follow the spatial regulation of fast molecular reactions occurring in the cell.

Knock-in fluorescent tags in genes of lower organisms, such us yeasts, has been previously used to achieve endogenous expression of fluorescent fusion proteins in order to measure biochemical parameters (Maeder et al., 2007). However, genome editing of mammalian cells has been more difficult to carry out, particularly in primary cells. Despite such challenge, a previous work already showed the power of genome editing to express fluorescent fusion proteins at endogenous levels in order to study molecular dynamics in mammalian cells (Doyon et al., 2011). This work evidenced the risk taken when protein localization or dynamics is evaluated under overexpression protocols *vs* the more reliable data obtained by monitoring fluorescent fusion proteins expressed at endogenous levels (Doyon et al., 2011).

Recently, genome editing by clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) has allowed *ad hoc* modification of genes in mammalian primary cells or cell lines with easy, fast, and highly efficient protocols (Mojica and Montoliu, 2016). Different contributions have shown the significance that endogenous expression of fluorescent fusion proteins has for biochemistry and cell biology (Fig. 1). For example, a fluorescent tag can help to identify molecular partners (Ruthig et al., 2021) or to monitor changes in localization occurring in response to cellular perturbations (Reicher et al., 2020). Nanoscale organisation and single molecule tracking of fluorescent fusion proteins has also been studied by using STED (stimulated emission depletion) microscopy (Willems et al., 2020). Moreover, single molecule imaging by PALM (photoactivated localization microscopy) has proved to be more accurate for fluorescent fusion proteins expressed at endogenous levels by CRISPR/Cas9 genome editing than for

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fluorescent fusion proteins overexpressed by traditional methods (Khan et al., 2019).

Beyond these approaches, which have helped to improve classical biochemistry and cell biology, quantitative biochemical parameters in mammalian cells have been measured. For example, a recent study reported the detection of fast diffusing subpopulations of protein compartmentalized in the cytosol or at cell membranes by combining CRISPR/Cas9-mediated genome editing and FCS (fluorescence correlation spectroscopy) (Castro-Sanchez et al., 2020). Likewise, a similar approach based on genome editing and FCCS (fluorescence cross-correlation spectroscopy) has been used to measure the dissociation constants (K_d) between two proteins, and their changes in response to physiological stimulation (Fig. 1) (Komatsubara et al., 2019; Eckert et al., 2020). FCS and FCCS enable us to measure the diffusion coefficient and concentration of endogenous proteins frequently expressed at very low levels. The insertion of tandem repeats of the fluorescent protein or the design of scaffolds to bind several copies of the fluorescent protein have been proven to be useful approaches to get enough signal in the case of low endogenous expression (Maeder et al., 2007; Tanenbaum et al., 2014; Rugbjerg et al., 2015). Remarkably, the expression of tandem chromophores with different sensitivity to a particular subcellular environment has been proven to be useful to track the traffic of molecules between different intracellular compartments (Jia and Bonifacino, 2019).

Thus, CRISPR/Cas9 genome editing is a turning point in cell biology and its use to develop endogenous fluorescent fusion proteins suitable to explore molecular reactions in live cells will aid to avoid the drawbacks associated with overexpression. We predict that the combination of edited cells with sensitive, quantitative and high-resolution microscopy will strongly push forward cell biology in the near future, not only for basic science but also for studies directed to understand the molecular perturbations occurring in cells treated with therapeutic drugs.

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CRediT authorship contribution statement

Carlos Carrasco-Padilla made the figure, contributed to article content and bibliography and revised the manuscript. Pedro Roda-Navarro

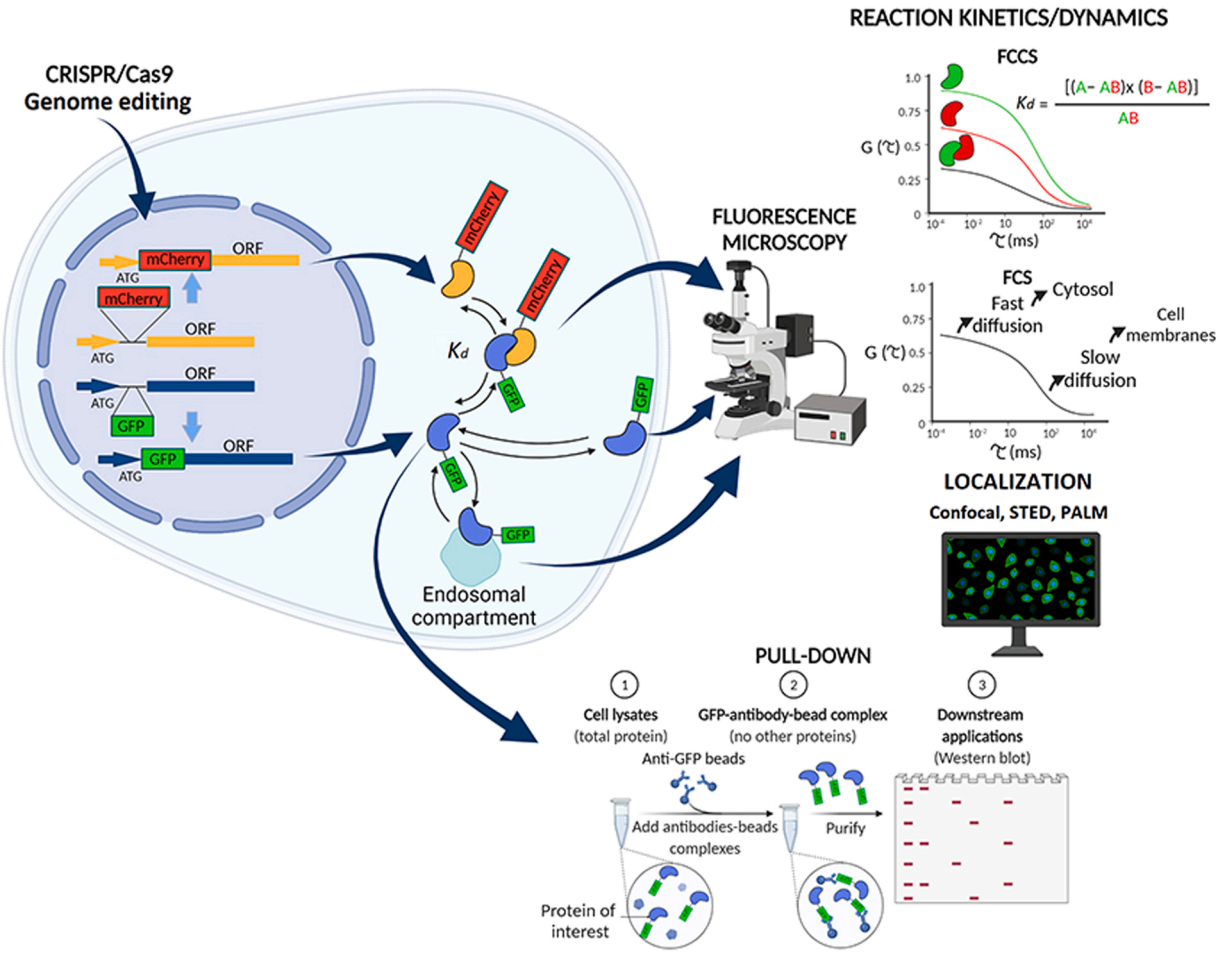

Fig. 1. Genome editing and molecular dynamics. CRISPR/Cas9 genome editing enables us to knock-in a fluorescent tag, such as the green fluorescent protein (GFP) or the monomeric (m)Cherry, in frame with the open reading frame (ORF) of particular genes. The endogenously encoded fluorescent fusion proteins can then be imaged under the fluorescence microscope to study reaction kinetics, dynamics and localization or used in classical biochemistry, for example in the identification of partners via pull-down of GFP-tagged molecules. In the FCCS representation, green and red autocorrelation curves resulted from the diffusion of total green-labelled (A) and red-labelled (B) proteins. The black line represents the cross-correlation due to A/B interaction. The equation for K_d estimation based on free and interacting protein concentration is indicated. In the FCS representation, diffusion pools can be deduced from complex autocorrelation curves. STED: stimulated emission depletion; PALM: photoactivated localization microscopy; FCS: fluorescence correlation spectroscopy; FCCS: fluorescence cross-correlation spectroscopy. Created with Biorender.com.

obtained funds for research and conceived and wrote the manuscript.

Declaration of Competing Interest

Authors have no competing Interest to declare.

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