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Probing labeling-induced lysosome alterations in living cells by imaging-derived mean squared displacement analysis

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ABSTRACT

Lysosomes are not merely degradative organelles but play a central role in nutrient sensing, metabolism and cell-growth regulation. Our ability to study their function in living cells strictly relies on the use of lysosome-specific fluorescent probes tailored to optical microscopy applications. Still, no report thus far quantitatively analyzed the effect of labeling strategies/procedures on lysosome properties in live cells. We tackle this issue by a recently developed spatiotemporal fluctuation spectroscopy strategy that extracts structural (size) and dynamic (diffusion) properties directly from imaging, with no *a-priori* knowledge of the system. We highlight hitherto neglected alterations of lysosome properties upon labeling. In particular, we demonstrate that Lipofectamine reagents, used to transiently express lysosome markers fused to fluorescent proteins (FPs) (e.g. LAMP1-FP or CD63-FP), irreversibly alter the organelle structural identity, inducing a ~2-fold increase of lysosome average size. The organelle structural identity is preserved, instead, if electroporation or Effectene are used as transfection strategies, provided that the expression levels of the recombinant protein marker are kept low. This latter condition can be achieved also by generating cell lines stably expressing the desired FP-tagged marker. Reported results call into question the interpretation of a massive amount of data collected so far using fluorescent protein markers and suggest useful guidelines for future studies.

1. Introduction

Lysosomes are sub-micrometric, dynamic, membrane-enclosed sub-cellular organelles. They represent the main degradative compartments of the cell [1], but have recently been proposed as signaling hubs, with a central role in a number of crucial processes, from nutrient sensing to metabolism and cell growth regulation [2,3]. As for many other sub-cellular structures/compartments, it is now well appreciated that the simultaneous tight control of lysosome structural (e.g. size) and dynamic (e.g. diffusivity, mode of motion) properties is key to their function in the cell, in physiology and in pathology [4–7]. In spite of the huge research efforts, retrieving quantitative, simultaneous access to both structural and dynamic information on such tiny subcellular structures remains a challenge in the field. If electron microscopy (EM)-based analysis was pivotal to probe the finest (ultra)structural details of many sub-cellular structure, including lysosomes [8], that was inevitably done at the expense of dynamic information. On the other hand, standard optical microscopy tools shed new light onto the lysosome dynamic behavior and its regulation [9–11], but typically under-exploited the spatial dimension and, as a consequence, the structural information. To tackle these limitations, some of us recently proposed a fluctuation-based analytical tool (named *imaging*-derived Mean Square Displacement, or *i*MSD) that is able to simultaneously extract both structural and dynamic average parameters of the target object from simple time-lapse imaging, with no a-priori knowledge on the system, no need to extract trajectories, no need for complex labelling, and in live cells [12] (a brief overview of the method is also reported here as Supplementary Information). Thanks to this approach, large-scale fingerprinting of dynamic structures can be achieved, in a fast and robust way, at the whole-cell-population level. The potency was demonstrated in preliminary applications on a variety of biological/biomedical targets, from molecules [12,13] to sub-cellular structures/organelles [14,15]. Concerning the lysosome in particular, we already showed

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how the retrieved organelle structural and dynamic features change depending on the experimental parameters (e.g. time scale selected, temporal resolution, pixel size, etc.), thus identifying specific guidelines for lysosome proper imaging and *i*MSD-based analysis [15,16]. Still, an unavoidable requirement for optical-microscopy-based analyses in living cells is labelling the structure of interest by fluorescent probes. Concerning lysosomes, this is typically achieved by either targeting a fluorescent (organic) dye to the lumen of the lysosome (e.g. LysoTracker) or by covalently attaching a genetically-encoded fluorescent protein (FP) to a lysosomal protein marker [17]. This latter is then expressed upon transient or stable transfection of the corresponding plasmid by means of standard procedures, such as lipofection or electroporation. In spite of the large amount of data produced in live cells on lysosomes following these procedures, little is known about the effect of standard labelling strategies on the average structural and dynamic properties of this organelle. We tackle this issue here by the *i*MSD approach.

To start, we benchmarked lysosome structural and dynamic features by using the standard fluorescent dye LysoTracker Red administered to HeLa cells following manufacturer's protocol. Time-lapse imaging of a region of interest ([17.28 × 17.28] μm) within the cell cytoplasm at a fixed total timescale (0–12 s) was performed (Fig. 1A). As explained in the Supplementary Information, spatiotemporal correlation analysis is performed on the acquired image stacks to calculate the average displacement of lysosomes. The whole population of retrieved *i*MSD traces is reported in Fig. 1B, together with the average trace (bold red line). *i*MSD fitting yielded a quantitative picture of the average lysosome structural and dynamic properties in our experiments. In particular, the measured σ_0^2 (y-axis intercept of the *i*MSD trace, used to estimate the organelle size, as previously demonstrated [15], and reported in Supplementary Information), D_m (local diffusivity), and α coefficient (anomalous diffusion coefficient) are reported, in the form of Mean ± SD values, in Fig. 1C, red squares (see also Table S1). As expected, results are in line with our previous observations [15,16], and with available literature [18]. These benchmark values were further tested by probing expected variations of lysosome size and dynamics. Concerning size, cells were treated with 50 μM Sucrose and then imaged using Lysotracker. Since Sucrose is not degraded within the lysosome, it is accumulated and induces an osmotic-swelling effect on the organelle [11]. In fact, *i*MSD analysis highlights an average increase (approximately 100 nm) in lysosome size upon Sucrose treatment (Fig. 1C, white squares and Table S1), with a concomitant minor effect on dynamics. Concerning dynamics, lysosome local diffusivity (D_m) was dramatically reduced upon ATP depletion (Fig. 1C, black squares and Table S1), with no effect on lysosome size. This matches previous results by Mithieux, G. & Rousset, B. [19], and is probably due to a more persistent interaction of lysosomes with microtubules in absence of ATP. Having probed the *i*MSD sensitivity to average variations of the lysosome structural and dynamic properties, we set out to study a number of lysosome-specific labeling strategies/procedures, which are routinely used worldwide. In particular, as mentioned above, we selected two specific protein markers of the lysosome, namely, LAMP1 and CD63. Their FP-tagged variants were introduced into cells by transfection, either transient (using Lifofectamine, Effectene, or electroporation) or stable, in HeLa cells. As an obvious control, we probed that lipofection resulted in the proper co-localization of both CD63-EGFP and LAMP1-GFP with the LysoTracker fluorescence signal (Suppl. Fig. 1, using Lipofectamine), in keeping with previous reports [11,20–23]. All tested conditions were compared to the Lysotracker-based benchmark by statistical meta-analysis on the three measured parameters, σ_0^2, D_m, and α, as reported in Fig. 2A–C. First, it is worth noting that Lipofectamine-driven transient expression of both LAMP1-GFP and CD63-EGFP yields clear structural alteration of lysosomes: a statistically significant average enlargement (1.6 folds, corresponding to ~250 nm) of the organelle is found in both cases at 24 h post-transfection, although with no consequences for lysosome dynamic properties (Fig. 2A–C, full blue and green circles, respectively). A similar effect is produced even if the time of exposure to Lipofectamine reagents is reduced to 4 h (see Suppl. Fig. 2). Moreover, such structural alteration of the lysosome is irreversible, as no recovery is observed even after 48 h of incubation in Lipofectamine-free medium (Suppl. Fig. 2). To test whether the lysosome structural alteration is caused by protein over-expression or by exposure to Lipofectamine reagents, an experiment was performed by the Lipofectamine-based transient expression of a lysosome-unrelated protein, i.e. untagged EGFP. Notably, Lipofectamine-driven expression of EGFP is associated with a clear enlargement of lysosomes (Fig. 2A, full black circle), almost to the same extent as described for LAMP1-GFP and CD63-EGFP. This result prompted us to test additional transfection reagents/procedures. In particular, we used Effectene (as an alternative lipid-based, but non-liposomal, method) and cell electroporation (in which an external electrical field is applied in order to transiently increase the permeability of cell membranes). It is worth noting that both methods are not producing lysosome alterations, neither structural nor dynamic, if associated with untagged EGFP (Fig. 2A–C, empty black circles and black stars, respectively). Quite surprisingly, however, both methods induce lysosome alteration (~1.5-fold increase in size, but negligible effect on dynamics) if associated with the expression of the two lysosome protein markers, LAMP1-GFP and CD63-EGFP (Fig. 2A–C, empty blue/green circles and blue/green stars). Taken together, these latter results, even if quantitatively similar to those obtained using Lipofectamine reagents, point to the effect of protein over-expression on the lysosome structural integrity. A number of additional control experiments and analyses were set to probe this hypothesis. First, two HeLa-based cell lines stably expressing either LAMP1-GFP or CD63-EGFP were generated in order to keep the recombinant protein expression at a minimum (see Materials and Methods for details). Notably, this was enough to recover the actual size of the lysosome (Fig. 2A, empty blue and green squares, respectively), irrespective of the specific protein marker used. At this point, in order to clarify whether lysosome structural alterations depend on the amount of protein accumulated on the lysosome membrane, we quantified the mean fluorescence intensity (MFI) of the whole population of imaged lysosomes per each cell in our transient transfection experiments and correlated MFI to the *i*MSD readouts. The scatter plots in Fig. 3 show the results obtained for LAMP1-GFP expressed under different transfection protocols. It is anticipated here that similar results were obtained with CD63-EGFP (as reported in Suppl. Fig. 3). Interestingly, the plot in Fig. 3A clearly demonstrates that lysosome structural alteration under Lipofectamine treatment is independent of the protein final concentration obtained on the organelle membrane (i.e. no significant correlation between MFI and apparent lysosome size in LAMP1-GFP expressing cells is found, fit slope ~0). By contrast, if LAMP1-GFP expression is triggered by Effectene- or electroporation-based procedures, a clear positive correlation is observed between the MFI and the extent of lysosome enlargement (Fig. 3B–C, fit slope>1): i.e. the higher the LAMP1-GFP expression level, the higher the average size of lysosomes. Please note that low expression levels of LAMP1-GFP (lower-left portion of the plots in Fig. 3B–C) yield an average lysosome size close to that of the Lysotracker control (the y-axis intercepts of the linear fits correspond to ~420 nm for Effectene and ~520 nm for electroporation) and to that retrieved from stably-transfected cell lines (the range of observed MFIs for these latter is indicated in all plots by a grey

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