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Single particle tracking of internalized metallic nanoparticles reveals heterogeneous directed motion after clathrin dependent endocytosis in mouse chromaffin cells

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Abstract

Most accepted single particle tracking methods are able to obtain high-resolution trajectories for relatively short periods of time. In this work we apply a straightforward combination of single-particle tracking microscopy and metallic nanoparticles internalization on mouse chromaffin cells to unveil the intracellular trafficking mechanism of metallic-nanoparticle-loaded vesicles (MNP-V) complexes after clathrin dependent endocytosis. We found that directed transport is the major route of MNP-Vs intracellular trafficking after stimulation (92.6% of the trajectories measured). We then studied the MNP-V speed at each point along the trajectory, and found that the application of a second depolarization stimulus during the tracking provokes an increase in the percentage of low-speed trajectory points in parallel with a decrease in the number of high-speed trajectory points. This result suggests that stimulation may facilitate the compartmentalization of internalized MNPs in a more restricted location such as was already demonstrated in neuronal and neuroendocrine cells (Bronfman *et al* 2003). Although further experiments will be required to address the mechanisms underlying this transport dynamics, our studies provide quantitative evidence of the heterogeneous behavior of vesicles mobility after endocytosis in chromaffin cells highlighting the potential of MNPs as alternative labels in optical microscopy to provide new insights into the vesicles dynamics in a wide variety of cellular environments.

Introduction

Adrenal chromaffin cells are important regulators of critical physiological parameters as cardiac frequency, metabolic rate, vasoconstriction and vasodilation during rest and especially during stress conditions. These neuroendocrine cells release adrenaline, noradrenaline and neuropeptides in response to action potentials firing, which is regulated by sympathetic cholinergic terminals of the splanchnic nerve (De Diego *et al* 2007). These chemical messengers are stored inside thousands of dense core secretory vesicles distributed in the cell cytoplasm. The fusion of these vesicles to the plasma membrane and the consequent release of those messengers are triggered by Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCCs) activated during action potentials (Augustine and Neher 1992).

The maintenance of secretion along time requires the continuous refilling of the releasable pool of vesicles at rates that match the exocytotic activity (Smith *et al* 1998, Sorensen 2004, Voets *et al* 1999). There is also accumulated data showing that different types of endocytosis contribute to vesicle replenishment (Wu *et al* 2014, Chan and Smith 2001, Moya-Díaz *et al* 2016). The mobilization of reserve vesicles to the plasma membrane as well as vesicle recycling after endocytosis require vesicle trafficking through the cytoplasm. It is well documented that actin microfilaments and microtubules play important roles in these processes (Trifaro *et al* 2008, Giner *et al* 2007, Giner *et al* 2005, Neco *et al* 2003, Merrifield *et al* 1999, Qualmann and Kelly 2000, Ceridono *et al* 2011). Although the use of total internal reflection fluorescence microscopy gave important insight into secretory vesicles mobilization in the narrow space close to plasma membrane (Steyer and Almers 1999), the characteristics of the vesicle trafficking occurring deeper in the cytosol are mostly unknown. A previous study shows that switches between different types of motion are present in the trajectory of vesicles after endocytosis in synaptic terminals (Joensuu *et al* 2016). The goal of this study was to investigate the intracellular dynamics of endocytic vesicles in the cytosol of mouse chromaffin cells by tracking individual vesicles for long periods of time after endocytosis. Unfortunately, the trajectory total length and the spatial resolution of image-based single particle tracking (SPT) methods by means of organic molecules is usually limited to a few seconds and tens of nanometers (depending on the number of photons a fluorescent molecule can emit before photobleaching), respectively (Manzo and Garcia Parajo 2015, Zhou and Wang 2010). This problem can be overcome by the use of metallic nanoparticles as alternative probes for SPT (Kusumi *et al* 2005).

Because of their strong brightness and imaging durability, metallic nanoparticles (MNPs) have been introduced as alternative labels in fluorescence microscopy (Tucker-Schwartz *et al* 2014, Estrada and Gratton, Aaron *et al* 2007, Wang *et al* 2005, Jain *et al* 2008, Aslan *et al* 2005, Anzalone *et al* 2015).

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This is in part because, unlike common fluorescent probes, such as organic dyes (Resch-Genger *et al* 2008) and quantum dots (Qdots) (Pinaud *et al* 2010), MNPs do not photobleach upon continuous illumination. On the other hand, photoinduced luminescence from gold NPs is efficiently generated using a femtosecond pulse laser source with excitation intensities lower than those used for two-photon imaging of living cells. It has been demonstrated that gold exhibits ~ 100 times more luminescence when irradiated with a two-photon excitation instead of one-photon excitation source. These unique properties of metallic NPs have already been applied by several authors such as Durr and collaborators (Durr *et al* 2007) who have shown that the two-photon-induced signal from cancer cells targeted with gold nanorods is three orders of magnitude brighter than the two-photon autofluorescence from the unlabelled cells. These characteristics make gold NPs attractive contrast agents for cell imaging and especially interesting in tracking experiments where an increase in the tracking time is desired. The use of single particle tracking (SPT) (Manzo and Garcia-Parajo 2015, Zhou and Wang 2010) and MNPs in combination provides a non-invasive, spatially localized, *in vivo* characterization for many biological systems. In this work, we induced the internalization of MNPs by two different methods, during 24 hours in rest conditions to allow significant constitutive endocytosis, and by 5 minutes high potassium depolarization to induce Ca^{2+} dependent exocytosis and subsequent endocytotic membrane compensation (Perez Bay *et al* 2007). As could be expected, our results show that the formation of metallic nanoparticle-loaded vesicle (MNP-V) is much more efficient when induced with high K^+ in comparison to constitutive endocytosis. Moreover, formation of MNP-Vs was strongly inhibited by the clathrin assembly inhibitor chlorpromazine. When in the cytosol, we applied a single particle tracking (SPT) method to study the MNP-V motion, and two-photon excitation microscopy (TPEM) to identify their location inside the cell. From the MNP-V's trajectories measured, we calculated the mean square displacements (MSD) to distinguish their type of motion and to calculate their instantaneous speed along the trajectory. This analysis shows that directed movement dominates vesicle trafficking in the cytosol after endocytosis and, surprisingly, the application of a depolarization stimulus during the tracking diminished the speed of such a movement. Finally, we used an actin-disrupting reagent to investigate the role of the actin network in the intracellular trafficking. These experiments showed that, after treatment with cytochalasin-D (Cyt-D), the fraction of directed trajectories decreased with respect to untreated cells. The results indicate that actin filaments are in part responsible for directed motion of vesicles, and suggest that stimulation confines MNPs in a less mobile compartment.

MATERIALS AND METHODS

Mouse adrenal chromaffin cell preparation and experimental solutions

All animal procedures were performed under protocols approved by the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and the Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires), and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication 80-23/96), USA, and local regulations.

Adrenal glands from two 13-18 days old female/male 129/sv mice were used in each culture. Chromaffin cells were isolated and cultured following the procedures described by Perez Bay et al (Perez Bay *et al* 2012). Briefly, mechanically isolated adrenal medullas were digested for 25 minutes in Hanks medium (see description below) with papaine (0.5-1 mg/ml) at 37 °C, and disrupted with a micropipette 20 µl tip in Dulbecco's modified Eagle's medium low glucose, supplemented with 5% fetal calf serum, 5 µl/ml penicillin/streptomycin, 1.3 µl/ml gentamicin, 1 mg/ml bovine sero albumin, and 10 µM cytosine-1-β-D-arabinofuranoside. The cell suspension was filtered through 200 µm and 50 µm pore meshes, and cultured on poly-L-lysine pretreated coverslips at 37 °C, 95% O₂ - 5% CO₂.

The Hanks medium used for cell culture contained (in mg/ml): 0.4 KCl, 0.06 KH₂PO₄, 8 NaCl, 0.35 Na₂CO₃, 0.09 Na₂HPO₄, supplemented with 3 µl/ml penicillin /streptomycin and 1 mg/ml of glucose. The standard solution used for the experiments contained 145 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 10 mM HEPES, 2 mM CaCl and 10 mM dextrose; pH = 7.2. To stimulate the cells we added 50 mM KCl in replacement of NaCl; keeping a constant osmolarity.

Three dimensional tracking microscope

A home-made two-photon instrument based on an inverted IX71 Olympus microscope was used for the measurements of SPT trajectories (Figure 1). For excitation, we used a mode-locked 80 MHz Ti:Sapphire laser (KMLabs, USA) pumped with a CW 5W-Verdi (Coherent). Laser pulses were 50 fs (FWHM), laser average power intensity at the microscope entrance ranged from 5 to 80 mW and excitation wavelength was set up at 800 nm for all the experiments. We used an UPlanFL N 60X 0.65 NA air objective (Olympus, Japan) and a short-pass dichroic mirror (680DCSPXR, Chroma Technology, USA) to direct the excitation light to the sample.

Additionally, a BG39 band-pass filter (Schott, Germany) was positioned after the dichroic mirror to filter out in the wavelength range of the Ti:Sapphire red emission. Three dimensional scanning was obtained using galvano motor-driven scanning mirrors (General Scanning, VM500), and a PIFOC P-

The diagram illustrates a pump-probe experimental setup. A **Pump laser** provides energy to a **Ti:Sapphire laser**. The output of the Ti:Sapphire laser is split into two paths. One path, representing the pump beam with a pulse width of ~ 50 fs, is directed through a **GM** (Galvanometer/Motor) unit and a **Dichroic mirror** to a **Sample** mounted on a **PZT or ETL** (Piezoelectric Transducer/Electro-Optic Transducer) stage. The other path, representing the probe beam with a pulse width of ~ 12.5 ns, is directed through a **Band-pass filter** to a **PMT** (Photomultiplier Tube). The sample is imaged by a **60X Objective** lens within an **Olympus IX-71 microscope**. The signal from the PMT is amplified by an **Amp** (Amplifier), then passed through a **Discriminator** to a **DAQ computer** (Data Acquisition computer). The DAQ computer also receives feedback from the GM unit.

exocytosis-endocytosis cycling. In order to do that, and before starting with the study of MNP-Vs cytosolic mobility, we compared the amount of internalized MNP-Vs in two conditions. The cells were always incubated in presence of a suspension of 10 nm gold MNPs at 30 pM, but in one group of experiments they were stimulated with extracellular 50 mM K⁺ solution, and in the other group they were incubated in standard solution (see Materials and Methods section) to keep cells in rest conditions. The endocytosis under rest conditions is in general associated to membrane components turnover, and is regularly identified as constitutive endocytosis. On the other hand, it is known that a high extracellular K⁺ application provoked massive exocytosis and subsequent vesicle endocytosis in mouse chromaffin cells (Perez Bay *et al* 2007). We called this type of endocytosis as high K⁺ induced endocytosis (HKE) to differentiate it from the constitutive endocytosis (CE). We applied HKE protocol in the presence of a gold NPs at 30 pM for 5 minutes, then we washed the cells with standard solution, and finally performed a z-stack of images to count the internalized particles in the whole cell volume. After analyzing 25 cells from 3 independent cell cultures, 16.9 ± 1.5 MNP-Vs per cell were obtained in HKE condition (Figure 2(a), HKE). Besides that, several hours were needed to obtain a measurable MNP-Vs formation when in CE. Therefore, we decided to fix the MNPs incubation time in rest conditions to 24 hours. The results in Figure 2(a) (CE) show that, even after that long incubation time, the formation of MNP-Vs in CE was 7 ± 1 MNP-s per cell, which is significantly low compared to the internalization obtained for HKE.

It is important to point out that the application of high K⁺ during 5 minutes promotes the exocytosis and subsequent endocytosis of a huge amount of membrane, equivalent to more than half of the cellular surface (Perez Bay *et al* 2007; Perez Bay *et al* 2012). In terms of secretory vesicles, it corresponds to more than 3000 vesicles/cell. On the other hand, the same treatment induces the internalization of 16.9 ± 1.5 MNP-Vs/cell. This extremely low efficiency to internalize MNPs during HKE is in fact an advantage, allowing us to easily identify and track single particles in a non-crowded environment, as it will be described in the next sections of this work.

Secondly, we evaluated if MNP-Vs internalization depends on classical clathrin-dependent endocytosis. With this aim, we pre-treated cells with 15 μ M chlorpromazine (CPZ) for 5 minutes before NPs internalization in HKE conditions, maintaining the drug during the whole experiment. CPZ is an inhibitor of clathrin layer assembly into the membrane that consecutively stops the formation of coated pits (Wang *et al* 1993). The results show a significant decrease in MNP-Vs formation in presence of CPZ (Figure 2(a), HKE+CPZ), indicating that the internalization of MNPs during HKE is at least partially produced via a clathrin-dependent endocytosis mechanism.

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Figure 2: Internalization of 10 nm gold nanoparticles in CE, HKE and HKE+CPZ conditions. (a) Amount of particles counted from a z-stack of two-photon images acquired after 24hs (CE) or 5 minutes (HKE and HKE+CPZ). (b) Fraction of internalized particles per minute. n = 19, 25 and 26 cells for CE, HKE and HKE+CPZ conditions respectively. At least three independent cell cultures were analyzed in each case. *** p<0.0001. CE: constitutive endocytosis, HKE: high potassium induced endocytosis, CPZ: chlorpromazine.

For a clear comparison among the amount of internalized particles in CE, HKE and HKE+CPZ conditions, Figure 2(b) shows the internalization rate as the amount of MNPs internalized per minute, being 0.0048 ± 0.0008 , 3.4 ± 0.3 , and 0.55 ± 0.09 MNP-Vs min^{-1} , for CE, HKE and HKE + CPZ respectively (p-value<0.0001).

Single-Particle Tracking of intracellular vesicles

Single particle tracking of internalized MNP-Vs were carried out to monitor and characterize the changes in their intracellular mobility taking place before and upon stimulation.

Briefly, the orbital tracking method (OTM) (Hellriegel and Gratton 2009, Levi *et al* 2005, Kis-Petikova and Gratton 2004) allows us to follow single entities while moving in three dimensions inside cells. In the OTM the observation volume of a two-photon or confocal microscope orbits around a moving object, a MNP-V in this work, keeping it centered through a feedback algorithm. This approach brings some advantages in comparison to the image-based single-particle tracking techniques. As has already been shown (Hellriegel and Gratton 2009, Levi *et al* 2005, Kis-Petikova and Gratton 2004), the OTM has a very high spatio-temporal resolution (1–50 nm, depending on the number of collected photons, and 1–30 ms depending on the brightness of the tracked particle and the time response of the

galvano/piezo devices).

First, transmission and two-photon excitation microscopy (TPEM) were used to recognize a cell and internalized MNP-Vs in the observation field. Vesicles internalized by HKE and located within $1.5\ \mu\text{m}$ of the cell border were identified and their trajectories measured for 1-5 minutes (pre-stimulus trajectory). This region includes the vast majority of vesicles internalized in our conditions, which is in agreement with previous reports describing the localization of internalized vesicles few minutes after high K^+ application (Patzak and Winkler 1986). Then, a second high K^+ stimulus was applied while still tracking (post-stimulus trajectory) leading to a total tracking time of 5 to 10 minutes. Figure 3 shows an example of a two-photon image of a chromaffin cell simultaneously with a contour drawing of the same cell and a representative measured trajectory. The black line in (b) represents the position of the cell border, while the internal blue line corresponds to the measured trajectory. All trajectories were measured with 8 – 32 ms temporal resolution, which corresponds to 60 – 250 times the resolution available in previous works (Maucort *et al* 2014). Because of the short displacement that was recorded in the z direction, a 2D tracking was sufficient to correctly follow the motion of the vesicle without losing it. For analysis purposes, the trajectory starting position is marked.

Figure 3: (a) Representative two-photon excitation microscopy image of a chromaffin cell with MNP-Vs identified as diffraction-limited bright spots. White arrow indicates the tracked vesicle. (b) Contour drawing of the same cell with mapped trajectory of previously indicated vesicle. Scale bar in panels (a) and (b) is 10 microns. (c) An expanded view of the measured trajectory shown in panel (b). The red arrow indicates the position where the stimulus was applied.

A visual inspection of the entire trajectory does not show differences between the type of motion before and after the stimulation. In fact, all (pre- and post-stimulus) trajectories suggest that MNP-Vs movement is characterized by directed motion. Certainly, a simple visual inspection of the trajectory is

not enough to undoubtedly identify the particles type of motion (Saxton 1993). The simplest and probably still most widely used approach to characterize intracellular transport phenomena is the Mean Square Displacement (MSD) analysis. However, previous experimental (Arcizet *et al* 2008, Mahowald *et al* 2009) and theoretical (Loverdo *et al* 2008) studies suggest that in many biological situations intracellular transport can be described as a combination of Brownian diffusion interspersed with periods of directed transport (Brangwynne *et al* 2009). Moreover, particles can switch between the two motility modes (Loverdo *et al* 2008). As a consequence, this complex dynamics cannot be resolved by a standard global MSD analysis of the whole trajectory. As a first approach and to compare the macroscopic vesicle mobility with previously reported results (Maucort *et al* 2014), a trajectory segmentation was performed by dividing the whole trajectory in arbitrary 1-minute-long sub-trajectories. After this segmentation procedure, we computed the individual MSDs from the obtained sub-trajectories, fitting the data with standard models to distinguish the kind of motion, and evaluated the dynamic parameters (flow speed and diffusion coefficient). Since the numerical computation of the MSD intrinsically suffers the low statistical significance at long lag times, τ , we restricted our analysis to τ values lower than 25% of the total track's time, in agreement with common SPT criterions (Saxton 1997, Monnier *et al* 2012, Ruthardt *et al* 2011). Figure 4 shows a typical MSD analysis of one representative track of a MNP-V internalized in HKE conditions, before (a) and after (b) the application of high K^+ stimulation. In this example the entire trajectory was divided in 5 sub-trajectories before (and 6 sub-trajectories after) the stimulation.

Figure 4: Mean square displacement (MSD) analysis of one representative track of a MNP-V in HKE condition, before (a) and after (b) the application of high K^+ stimulation. The trajectories were segmented in 1-minute-long sub-trajectories and individual MSDs calculations were performed to assess the type of motion. Colors in MSD curves correspond with those in the time-coded vesicle trajectory (insets). The best curve fittings are also shown (black solid line). After the MSD analysis

of all pre- and post-stimulus sub-trajectories 92.6% in 68 sub-trajectories best fitted to a quadratic polynomial (with $R^2 > 0.999$), describing a directed type of transport of MNP-Vs.

After the MSD analysis of all pre- and post-stimulus sub-trajectories, we are able to conclude that directed transport is the major route of MNP-Vs intracellular trafficking (92.6% in 68 sub-trajectories obtained after HKE). It is important to remark that the MSD analysis as was performed in this work might not be enough to determine the positions where the MNP-V's mobility changes along the trajectory. Further segmentation of trajectories in shorter intervals can be necessary, but this characterization is not in the goals of the present work.

Finally, as exemplified in Figure 3(b), most of the vesicles located within 1.5 μm of the cell border were moving towards the cell center. This tendency was repeated in 70% of cells analyzed. This result together with the sub-trajectories MSD analysis (Figure 4), suggests that after endocytosis MNP-Vs are moving away from membrane through a macroscopic directed mode of motion.

Taking advantage of the enhanced temporal resolution of our tracking method (time between consecutive points along the trajectory ranged from 8 to 32 ms), to further analyse the MNP-V' motion we then focused our work on the study of their instantaneous speed (modulus of the instantaneous velocity), at a single vesicle level. We calculated the instantaneous speed, computed as the ratio of the particle displacement (distance between 2 consecutive points in the trajectory) and the time of a single step, at all the trajectory points. Figure 5(a) shows the pre- (in blue) and post-stimulus (in red) speed distributions obtained from the same trajectory of MNP-Vs internalized by HKE represented in Figure 4. An increase in the percentage of low-speed trajectory points was obtained in the post-stimulus trajectory time interval, in parallel with a decrease in the number of high-speed trajectory points. A Mann-Whitney test (Hart 2001, Nachar 2008) analysis confirms that after the application of the extracellular K^+ stimulus all MNP-Vs internalized by HKE became significantly less mobile at a significance level of 0.05 (Bonferroni corrected). On the contrary, for the MNP-Vs internalized by CE only 50% of the post-stimulus trajectories showed a reduction in their median speed (Figure 5(b); Mann-Whitney test not significant for any trajectory at a Bonferroni-corrected 0.05 significance level).

Figure 5: Instantaneous speed distributions of two representative trajectories for vesicles internalized by HKE (a) and CE (b) conditions. Instantaneous speed was calculated at all the trajectory points in each recorded pre- (in blue) and post-stimulus (in red) trajectories. (a) This representative example of a vesicle internalized by HKE shows an increase in the percentage of low-speed trajectory points in the post-stimulus trajectory region, in parallel with a decrease in the number of high-speed trajectory points. (b) In this example of a vesicle internalized by CE the pre- and post-stimulus speed's distributions were not statistically different. Statistical analysis of independent experiments was performed using the Mann–Whitney U test at a significance level of 0.05 (Bonferroni corrected). Values in both panels were normalized to the amount of calculated speeds in each trajectory ($n_{\text{speeds}} > 15000$).

At first, these results might appear counterintuitive. Since high K^+ stimulus activates voltage dependent Ca^{2+} channels provoking a maintained cytosolic Ca^{2+} increase during several seconds (Perez Bay *et al* 2012), it would be reasonable to expect an increase of directed vesicle movement through the cytoskeleton (Colombo *et al* 1997). However, one possible explanation is that stimulation facilitates some compartmentalization of internalized MNPs in a more restricted location such as an endosome or Golgi (Bauer and Neher 2004, Phillips *et al* 1983, Bronfman *et al* 2003, Maxfield and McGraw 2004, Knight 2002). Actually, it was already reported that in neuronal and neuroendocrine cells vesicles internalized by clathrin dependent endocytosis can fuse with endosomes (Bronfman *et al* 2003) and that this fusion is stimulated by cytosolic Ca^{2+} (Colombo *et al* 1997). On the contrary, CE internalized vesicles did not follow such a clear behavior, possibly as a consequence of the long incubation time needed for CE. Consequently, it is likely that CE internalized vesicles are already in a later cycling step.

Now the question arises on whether the distribution of instantaneous speeds along a measured trajectory was randomly distributed or involved in a more gradual pattern. To investigate this issue, we determined the speed between every two trajectory points and then painted them according to a color code. Figures 6(a), (c) and Figures 6(b), (d) show the velocity maps measured before (pre-) and immediately after (post-) stimulation respectively. Within each 5 to 10-minutes data acquisition, the MNP-V showed higher (warm colors in Figure 6) and lower (cold colors in Figure 6) values in their local speed. This analysis further revealed that the slowing down vesicles' speed shown in Figure 5 is not simply a behavior becoming progressive with time, and in fact reflects a vesicle-mobility heterogeneity, which may contain meaningful information at the single-vesicle level. We ignore, however, the possible biological implications of this heterogeneity, what remains as a challenge for future works.

Figure 6: Calculated instantaneous speeds along a trajectory (a and c) pre-, (b and d) post-stimulation. (a and b) is the xy projection of the calculated speeds along a trajectory. (c and d) to facilitate the visualization, a 3D plot is also shown simultaneously with a speed-coded axis where dark red indicates 14.5 nm/s and deep blue 0 nm/s. For reference, solid black line is the measured trajectory, and the black circle indicates the tracking starting position.

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It is known that the cytoskeleton plays a fundamental role in the internalization and trafficking of vesicles in response to stimulation (Neco *et al* 2003, Chen *et al* 2001, Wen *et al* 2011). We used this knowledge to build confidence on our technique and results by treating the cells with 2 μ M of cytochalasin D (Cyt-D) and comparing the obtained trajectories with the untreated cells. Cyt-D belongs to a class of actin-depolymerizing agents that specifically binds to actin filaments inhibiting its polymerization (Doreian *et al* 2008). As expected, Cyt-D treatment is able to disrupt the F-actin network, as was clearly visualized by fluorescence microscopy (data not shown). MNP-Vs were tracked in Cyt-D-treated cells, leading to 20 pre- and 20 post-stimulus sub-trajectories. Figure 7 shows that the percentage of non-directed sub-trajectories increased from 7.4% in untreated cells to 20% in Cyt-D-treated cells (68 and 40 sub-trajectories, of 13 and 9 cells respectively) suggesting that F-actin microfilaments are at least partially responsible for directed transport of secretory vesicles.

It is important to note that after Cyt-D treatment chromaffin cells still possess a fraction of F-actin filaments that may be functional, and also an undisturbed microtubule cytoskeleton. Thus, the endocytic vesicles showing directed trajectories after Cyt-D treatment might be moving along the remaining F-actin filaments and on microtubules, opening the possibility of the contribution of both

cytoskeletal elements. It is important also to consider that inside the area of analysis of 1.5 μm from the cell border the F-actin filaments conform the so called cortical actin, which is particularly dense in chromaffin cells and is important in the motion of secretory vesicles (Neco *et al* 2004).

Conclusions

Most accepted single particle tracking methods are able to obtain high-resolution trajectories for relatively short periods of time. This is partly because commonly used fluorescent probes usually suffer from undesirable photochemical processes, such as blinking and photobleaching, limiting their utilization as labels for fluorescence-based techniques. One of the main challenges in tracking experiments where an increase in the total tracking time is desired is to design higher stability against photobleaching alternative labels. Over the last decade, metallic nanoparticles have become popular as alternative labels for cell imaging but little attention has been given to use them closely with tracking methods to track events over long periods of time.

In this work, we studied the dynamics of metallic-nanoparticle-loaded vesicles generated by endocytosis after high K^+ depolarization in chromaffin cells. Previous studies described that vesicles follow a complex behaviour where Brownian, directed and constrained diffusion models may coexist. Our ability to follow the vesicle mobility for long periods of time, with high spatio-temporal resolutions, and during the application of the stimulus, reveals a new level of complexity: the variations in the instantaneous speed. Although further experiments to address the mechanisms underlying these transport dynamics will be required, our studies provide the first quantitative evidence of the heterogeneous behavior of vesicles mobility after endocytosis in chromaffin cells. These results highlight the potential of metallic nanoparticles as new labels to provide new insights into the vesicles dynamics in a wide variety of cellular models.

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References

- (Aaron *et al* 2007) Aaron J., Nitin N., Travis K., Kumar S., Collier T., Park S.Y., José-Yacamán M., Coghlan L., Follen M., Richards-Kortum R., and Sokolov K., *Plasmon resonance coupling of metal nanoparticles for molecular imaging of carcinogenesis in vivo*. J Biomed Opt. 2007, 12(3):034007.
- (Annibale *et al* 2016) Annibale P., Dvornikov A. and Gratton E. *Optical measurement of focal offset in tunable lenses*. Opt Express., 2016, 24(2):1031-1036.
- (Anzalone *et al* 2015) Anzalone A., Gabriel M., Estrada L.C. and Gratton E., *Spectral properties of single gold nanoparticles in close proximity to biological fluorophores excited by 2-Photon excitation*. PLoS ONE, 2015, 10(4): e0124975.
- (Arcizet *et al* 2008) Arcizet D., Meier B., Sackmann E., Radler J.O. and Heinrich D., *Analysis of active and passive transport in living cells*, Phys. Rev. Lett., 2008, 101, 248103.
- (Aslan *et al* 2005) Aslan K., Lakowicz J. R. and Geddes C.D., *Plasmon light scattering in biology and medicine: new sensing approaches, visions and perspectives*. Curr. Opin. Chem. Biol., 2005, 9(5): 538-544.
- (Augustine and Neher 1992) Augustine G.J., and Neher E., *Calcium requirements for secretion in bovine chromaffin cells*. J. Physiol., 1992, 450, 247–271.
- (Bauer *et al* 2004) Bauer A.R., Overlease R.L., Lieber J.L. and Angleson J.K., *Retention and stimulus-dependent recycling of dense core vesicle content in neuroendocrine cells*, Journal of Cell Science, 2004, 117: 2193-2202.
- (Brangwynne *et al* 2009) Brangwynne C.P., Koenderink G.H., MacKintosh F.C. and Weitz D.A., *Intracellular transport by active diffusion*, Trends Cell Biol., 2009, 19, 423.
- (Bronfman *et al* 2003) Bronfman F.C., Tcherpakov M., Jovin T.M. and Fainzilber M., *Ligand-induced internalization of the p75 neurotrophin receptor: A slow route to the signaling endosome*, J. Neurosci., 2003, 23(8): 3209-3220.
- (Ceridono *et al* 2011) Ceridono M., Ory S., Momboisse F., Chasserot-Golaz S., Houy S., Calco V., Haeblerlé A.M., Demais V., Bailly Y., Bader M.F. and Gasman S., *Selective recapture of secretory granule components after full collapse exocytosis in neuroendocrine chromaffin cells*, Traffic, 2011, 12, 72–88.

- (Chan and Smith 2001) Chan S.A. and Smith C., *Physiological stimuli evoke two forms of endocytosis in bovine chromaffin cells*, J. Physiol., 2001, 537, (3): 871–885.
- (Chen *et al* 2001) Chen F., Rowen L., Hood L. and Rothenberg E.V. *Differential transcriptional regulation of individual TCR V beta segments before gene rearrangement*. J. Immunol., 2001, 166, 1771–1780.
- (Colombo *et al* 1997) Colombo M.I., Beron W. and Stahl P.D., *Calmodulin regulates endosome fusion*, J. Biol. Chem., 1997, 272, (12): 7707–7712.
- (De Diego *et al* 2007) De Diego A.M.G, Gandía L. and García A.G., *A physiological view of the central and peripheral mechanisms that regulate the release of catecholamines at the adrenal medulla*. Acta Physiol, 2007, 192, 287-301.
- (Doreian *et al* 2008) Doreian B.W., Fulop T.G. and Smith C.B., *Myosin II activation and actin reorganization regulate the mode of quantal exocytosis in mouse adrenal chromaffin cells*. J Neurosci., 2008, 28(17):4470-4478.
- (Durr *et al* 2007) Durr N.J., Larson T., Smith D.K., Korgel B.A., Sokolov K. and Ben-Yakar A. *Two-photon luminescence imaging of cancer cells using molecularly targeted gold nanorods*. Nano Lett., 2007, 7(4):941-945.
- (Estrada and Gratton 2012): Estrada L.C. and Gratton E., *Spectroscopic Properties of Gold Nanoparticles at the Single-Particle Level in Biological Environments*. Chem Phys Chem, 2012, 13(4): 1087-1092.
- (Giner *et al* 2005) Giner D., Neco P., del Mar Francés M., López I., Viniegra S. and Gutiérrez L.M., *Real-time dynamics of the F-actin cytoskeleton during secretion from chromaffin cells*. J Cell Sci., 2005, 118(13): 2871-2880.
- (Giner *et al* 2007) Giner D., López I., Villanueva J., Torres V., Viniegra S. and Gutiérrez LM., *Vesicle movements are governed by the size and dynamics of F-actin cytoskeletal structures in bovine chromaffin cells*. Neuroscience, 2007, 146(2): 659-669.
- (Hart 2001) Hart A., *Mann-Whitney test is not just a test of medians: differences in spread can be important*, BMJ. 2001, 323(7309): 391–393.

(Hellriegel and Gratton 2009) Hellriegel C. and Gratton, E. *Real-time multi-parameter spectroscopy and localization in three-dimensional single-particle tracking*. J. R. Soc. Interface, 2009, 6, Suppl 1, S3–14.

(Jain *et al* 2008) Jain P.K., Huang X., El-Sayed I.H. and El-Sayed M.A. *Noble metals on the nanoscale: optical and photothermal properties and some applications in imaging, sensing, biology, and medicine*. Acc Chem Res., 2008, 41(12):1578-1586.

(Joensuu *et al* 2016) Joensuu M., Padmanabhan P., Durisic N., Bademosi A.T., Cooper-Williams E., Morrow I.C., Harper C.B., Jung W., Parton R.G., Goodhill G., Papadopoulos A. and Meunier F.A. *Subdiffractional tracking of internalized molecules reveals heterogeneous motion states of synaptic vesicles.*, J Cell Biol., 2016, 215(2):277-292.

(Kis-Petikova and Gratton 2004) Kis-Petikova K. and Gratton E. *Distance measurement by circular scanning of the excitation beam in the two-photon microscope*. Microsc. Res. Tech., 2004, 63, 34-49.

(Knight 2002) Knight D.E., *Calcium-dependent transferrin receptor recycling in bovine chromaffin cells*. Traffic., 2002, 3(4): 298-307.

(Kusumi *et al* 2005) Kusumi A., Nakada C., Ritchie K., Murase K., Suzuki K., Murakoshi H., Kasai R.S., Kondo J. and Fujiwara T. *Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules*. Annu. Rev. Biophys. Biomol. Struct., 2005, 34, 351–378.

(Levi *et al* 2005) Levi V., Ruan Q., Plutz M., Belmont A.S. and Gratton E. *Chromatin dynamics in interphase cells revealed by tracking in a two-photon excitation microscope*. Biophys J., 2005, 89(6): 4275–4285.

(Loverdo *et al* 2008) Loverdo C., Benichou O., Moreau M., and Voituriez R. *Enhanced reaction kinetics in biological cells*, Nat. Phys., 2008, 4, 134.

(Mahowald *et al* 2009) Mahowald J., Arcizet D. and Heinrich D. *Impact of external stimuli and cell micro-architecture on intracellular transport states*, Chem Phys Chem, 2009, 10, 1559-1566.

(Manzo and Garcia Parajo 2015) Manzo C. and Garcia-Parajo M.F. *A review of progress in single particle tracking: from methods to biophysical insights*, Rep. Prog. Phys. 2015, 8, 124601.

(Maucort *et al* 2014) Maucort G., Kasula R., Papadopoulos A., Nieminen T., Rubinsztein-Dunlop H. and

Meunier F. *Mapping organelle motion reveals a vesicular conveyor belt spatially replenishing secretory vesicles in stimulated chromaffin cells*. PLoS ONE, 2014, 9(1): e87242.

(Maxfield and McGraw 2004) Maxfield F.R. and McGraw T.E., *Endocytic recycling*. Nat. Rev. Mol. Cell Biol., 2004, 5(2):121-132.

(Merrifield *et al* 1999) Merrifield C.J., Moss S.E., Ballestrem C., Imhof B.A., Giese G., Wunderlich I. and Almers W., *Endocytic vesicles move at the tips of actin tails in cultured mast cells*, Nat. Cell Biol., 1999, 1, 72-74.

(Monnier *et al* 2012) Monnier N., Guo S., Mori M., He J., Lénárt P. and Bathe M. *Bayesian approach to MSD-based analysis of particle motion in live cells*, Biophys J. 2012, 103(3): 616–626.

(Moya-Díaz *et al* 2016) Moya-Díaz J., Álvarez Y.D., Montenegro M., Bayonés L., Belingheri A.V., González-Jamett A.M., Cárdenas A.M. and Marengo F.D., *Sustained Exocytosis after Action Potential-Like Stimulation at Low Frequencies in Mouse Chromaffin Cells Depends on a Dynamin-Dependent Fast Endocytotic Process*. Front. Cell. Neurosci., 2016, 10, 184.

(Nachar 2008) Nachar N., *The Mann-Whitney U: A Test for Assessing Whether Two Independent Samples Come from the Same Distribution*, Tutorials in Quantitative Methods for Psychology, 2008, 4(1): 13-20.

(Neco *et al* 2003) Neco P., Giner D., del Mar Francés M., Viniegra S. and Gutiérrez LM., *Differential participation of actin- and tubulin-based vesicle transport systems during secretion in bovine chromaffin cells*. Eur J Neurosci. 2003, 18(4):733-42.

(Neco *et al* 2004.) Neco P., Giner D., Viniegra S., Borges R., Villarroel A. and Gutierrez L.M., *New Roles of Myosin II during Vesicle Transport and Fusion in Chromaffin Cells*, J Cell Biol., 2004, 279(26): 27450–27457.

(Patzak and Winkler 1986) Patzak A. and Winkler H. *Exocytotic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling*. J Cell Biol. 1986, 102(2):510-515.

(Perez Bay *et al* 2007) Perez Bay A.E., Ibanez L.I. and Marengo F.D., *Rapid recovery of releasable vesicles and formation of nonreleasable endosomes follow intense exocytosis in chromaffin cells*. Am J Physiol Cell Physiol, 2007, 293, C1509-1522.

- (Perez Bay *et al* 2012) Perez Bay A.E., Belingheri A.V., Álvarez Y.D. and Marengo F.D., *Membrane cycling after the excess retrieval mode of rapid endocytosis in mouse chromaffin cells*, *Acta Physiol* 2012, 204, 403–418.
- (Phillips *et al* 1983) Phillips J.H., Burrridge K., Wilson S.P. and Kirshner N., *Visualization of the exocytosis/endocytosis secretory cycle in cultured adrenal chromaffin cells*, *J Cell Biol*, 1983, 97(6):1906-1917.
- (Pinaud *et al* 2010) Pinaud F., Clarke S., Sittner A. and Dahan M., *Probing cellular events, one quantum dot at a time*. *Nat. Methods*, 2010, 7(4):275-85.
- (Qualmann and Kelly 2000) Qualmann B. and Kelly R.B., *Syndapin Isoforms Participate in Receptor-mediated Endocytosis and Actin Organization*. *J. Cell Biol*, 2000, 148(5): 1047–1061.
- (Resch-Genger *et al* 2008) Resch-Genger U., Grabolle M., Cavaliere-Jaricot S., Nitschke R. and Nann T., *Quantum dots versus organic dyes as fluorescent labels*, *Nat. Methods*, 2008, 5, 763 – 775.
- (Ruthardt 2011) Ruthardt N., Lamb D.C. and Brauchle C., *Single particle tracking as a quantitative microscopy-based approach to unravel cell entry mechanisms of viruses and pharmaceutical nanoparticles*. *Mol. Ther.*, 2011, 19(7):1199-1211.
- (Saxton 1993) Saxton M.J., *Lateral diffusion in an archipelago: Single-particle diffusion*. *Biophys. J.*, 1993, 64(6): 1766-1780.
- (Saxton 1997) Saxton M.J., *Single-particle tracking: the distribution of diffusion coefficients*. *Biophys. J.*, 1997, 72: 1744-1753.
- (Smith *et al* 1998) Smith C., Moser T., Xu T. and Neher E., *Cytosolic Ca²⁺ acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells*. *Neuron*, 1998, 20, 1243-1253.
- (Sorensen 2004) Sorensen J.B., *Formation, stabilisation and fusion of the readily releasable pool of secretory vesicles*. *Pflügers Archiv: European Journal of Physiology*, 2004, 448, 347-362.
- (Steyer and Almers 1999) Steyer J.A. and Almers W., *Tracking single secretory granules in live chromaffin cells by evanescent-field fluorescence microscopy*. *Biophys J*, 1999, 76, 2262–2271.
- (Trifaró *et al* 2008) Trifaró J.M., Gasman S. and Gutiérrez L.M., *Cytoskeletal control of vesicle transport and exocytosis in chromaffin cells*. *Acta Physiol (Oxf)*, 2008, 192(2):165-172.

- (Tucker-Schwartz *et al* 2014): Tucker-Schwartz J.M., Beavers K.R., Sit W.W., Shah A.T., Duvall C.L. and Skala M.C. *In vivo imaging of nanoparticle delivery and tumor microvasculature with multimodal optical coherence tomography*. Biomed Opt Express, 2014, 5(6): 1731-1743.
- (Voets *et al* 1999) Voets T., Neher E. and Moser T., *Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices*. Neuron, 1999, 23, 607-615.
- (Wang *et al* 1993) Wang L.H., Rothberg K.G. and Anderson R.G., *Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation*. J Cell Biol., 1993, 123(5): 1107-1117.
- (Wang *et al* 2005) Wang H.F., Huff T.B., Zweifel D.A., He W., Low P.S., Wei A. and Cheng J.X., *In vitro and in vivo two-photon luminescence imaging of single gold nanorods*. Proc. Natl. Acad., 2005, 44, 15752-15756.
- (Wen *et al* 2011) Wen P.J., Osborne S.L., Zanin M., Low P.C., Wang H.T., Schoenwaelder S.M., Jackson S.P., Wedlich-Söldner R., Vanhaesebroeck B., Keating D.J. and Meunier F.A., *Phosphatidylinositol (4,5) biphosphate coordinates actin-mediated mobilization and translocation of secretory vesicles to the plasma membrane of chromaffin cells*. Nat. Commun., 2011, 2, 491.
- (Wu *et al* 2014) Wu L.G., Hamid E., Shin W. and Chiang H.C., *Exocytosis and endocytosis: modes, functions, and coupling mechanisms*. Annu. Rev. Physiol., 2014, 76:301-31.
- (Zhou and Wang 2010) Zhou X. and Wang L., *Uses of single-particle tracking in living cells*, Drug Discoveries & Therapeutics. 2010, 4(2): 62-69.