1.3.23 Advanced microscopy and single-particle tracking: applications to neuroscience

We exploited, optimized, and developed various techniques in light-based microscopy for analyzing living matter at cellular and molecular levels. In particular, we developed a toolbox for single particle tracking (SPT) of membrane receptors and their ligands, suitable also for relatively fast single-pass membrane receptors; this is based on chemical tagging of recombinant proteins, TIRF microscopy, and automatized analyses of single-particle trajectories. We are now extending it to simultaneous visualization and analysis of two moieties.
The superresolved localization of this technique allowed analyzing dynamics, functions, interactions and stoichiometry of (pro)neurotrophin receptors $p75^{NTR}$ and TrkA and of their ligands in living cells.

Techniques in light-based microscopy continue to be central tools for analyzing organization, function and interactions of and within cells from the tissue down to the molecular levels. In particular, the methods of reconstruction of single-particle trajectories (single-particle tracking - SPT) are allowing the study of dynamics and transient interactions of molecules at excellent space-time resolutions, up to the order of (tens of) nanometers and milliseconds, and multicolor methods allow to analyze interactions between different objects in living cells [2]. Moreover, the SPT techniques are intrinsically super-resolved, and allow to obtain a distribution of the parameters of interest on the population; among these, the average lifetime of homo- and hetero-dimers, the constants of association and dissociation, their type of motion and, in case, their diffusivity [2].
We developed procedures for labeling, visualization and tracking of relatively fast-moving membrane receptors and their ligands, and methods to maximize the effectiveness of SPT data analysis, especially for the two-color analysis we are implementing. Proteins of interest are modified with peptidic tags and labelled by enzymatic reactions; we implemented and optimized short versions of this kind of tags that could also be functionalized orthogonally with different fluorophores [1, 5]. The possibility to label only receptors on the membrane with virtually any tag (e.g. fluorophores, quantum dots) is useful in various experiments [12]. Moreover, if needed, we can tune protein expression using inducible lentiviral vectors [1,8]. The movement of the receptors in the membrane and/or of the ligands within vesicles are then captured by (multi-channel) TIRF or epifluorescence microscopy.
Research on nervous system and neural cells is pivotal in our laboratory [6,7,10], and we applied also the SPT toolbox to the study of neurotrophins and their receptors [1-5,8,9]. Neurotrophic signaling is regulated through a complex interplay of different ligands and receptors, whose detailed network of interactions is still a source of debate. Superresolved SPT of receptors or ligands can help solving these issues, e.g. by counting the molecules in complexes while inspecting their dynamics and interactions in the membrane of living cells at low expression levels.
We studied dynamics, stoichiometry, and signaling of TrkA and $p75^{NTR}$ neurotrophic receptors (and some mutants of them) on the membrane of various cell lines and primary neurons or astrocytes (Fig. 1). This analysis, also after treatments with ligands or drugs, unraveled their mode of action in the first steps of sundry signaling pathways; indeed, the molecular basis for initiating signaling pathways can be very different, even if the same receptor and the same or similar ligands are involved. Moreover, we found puzzling results on commonly used control mutants [8, 9].

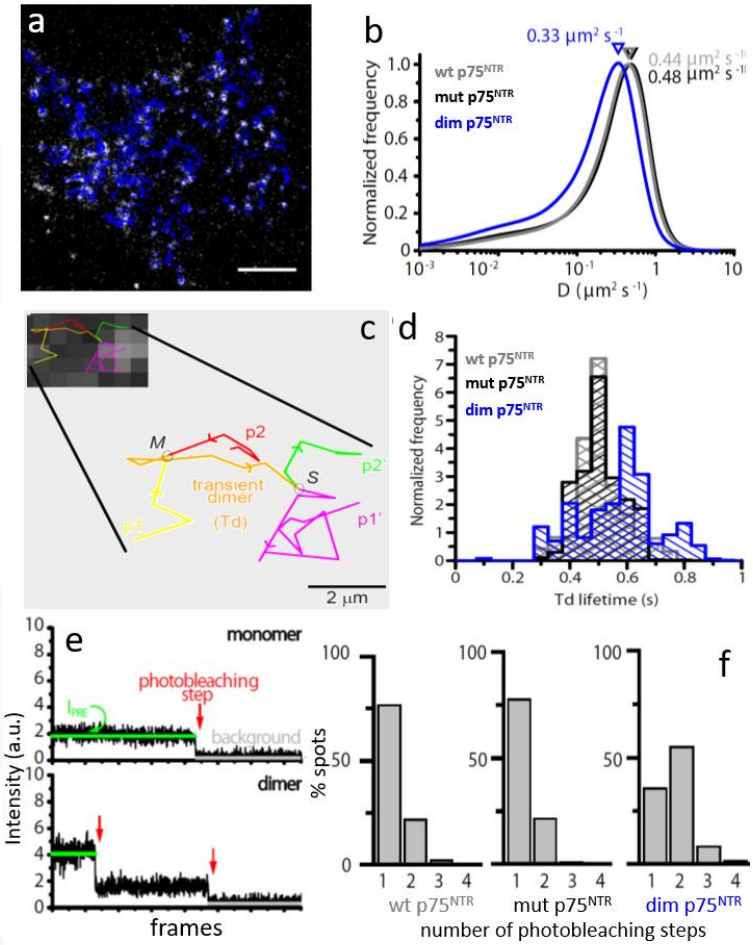

Figure 1. Examples of possible results from single particle tracking (SPT): application to neurotrophin receptors. **a-f)** Membrane p75^{NTR} moves as a monomer with at most transient interactions: membrane located wild type (wt) p75^{NTR}, a monomeric (mut p75^{NTR}) and a dimeric (dim p75^{NTR}) controls where labeled with Abberior635P exploiting a peptidic tag and imaged with TIRF microscopy. **a)** Reconstructed single receptor trajectories (blue) superimposed on a TIRF movie frame. Scale bar: 5 μm. **b)** Distribution of diffusion coefficient D for the constructs. **c-d)** Analysis of transient dimerization: **c)** transient dimer (Td) trajectory superimposed on a region of a TIRF movie frame and enlarged on bottom (M: merge, S: split events); **d)** distribution of the cell-average duration of Td trajectories for wt (gray), mut (black) and dim (blue) p75^{NTR} constructs. All data are from cells with [0.18-0.36] spots/μm^2. **e)** Examples of intensity profile traces of monomers and dimers. I_{PRE}: particle average intensity before the first bleaching step; red arrows: counted single photobleaching steps. **f)** Histograms of the number of photobleaching steps per trace for wt, mut and dim p75^{NTR}.

In particular, we contributed to solve some controversial results on stoichiometry and activation mechanisms for different signaling pathways of P75^{NTR} in the membrane of living cells (Fig. 2). We showed that, at densities of 0.2-0.5 receptors/μm^2, most receptors are fast diffusing monomers (D~1 μm^2/s in neurons), not substantially altered by (pro)neurotrophins administration. We found different signaling mechanisms for apoptosis and axon growth-cone collapse, and demonstrated that, in leaving cells at low expression levels, P75^{NTR} and a mutant developed as a monomeric control (mut P75^{NTR}) have the same stoichiometry in membrane, but a different partitioning into cholesterol-rich membrane regions upon NGF stimulation [8]. In a recent work, we studied

different TrkA kinase-dead mutants that were previously used for various work; we found that they can have different membrane trafficking and functions (among themselves and with respect to the wild type counterpart), and found that some of these differences are caused by different molecular structures; moreover, we demonstrated the existence of at least two distinct membrane immobilization modes, linked or not to NGF stimulation [9].

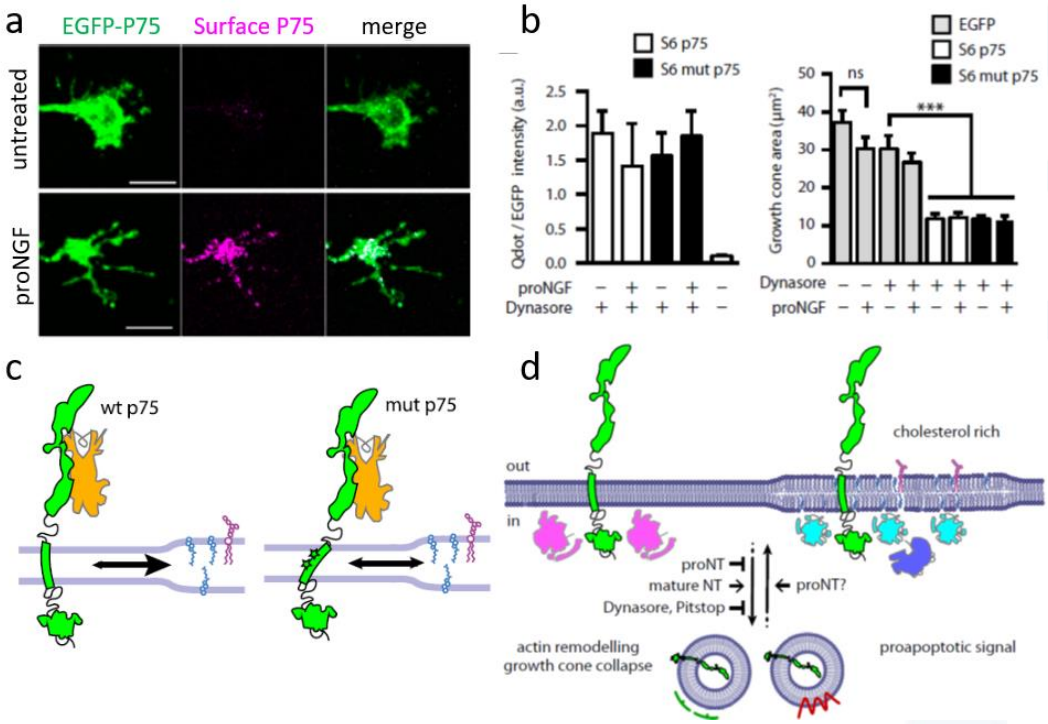

Figure 2. a-b) P75^{NTR} was fused with a fluorescent protein (EGFP), and only the receptors on the membrane were functionalized with biotin using a short peptidic tag (S6) and labeled with streptavidinated quantum dots (Qdot); the Qdot to EGFP ratio is a measure of surface p75NTR-EGFP fraction. **a)** Representative confocal images of growth cones of wt hippocampal neurons, transfected with S6-tagged p75^{NTR}-EGFP constructs, untreated (top) or incubated with proNGF for 30' (bottom). Total (green) and surface (magenta) receptor pools are shown. EGFP channel levels in the left panels are linearly scaled to highlight cone dimensions. Scale bar, 5μm. **b)** Left: both wt and mut p75^{NTR} are enriched on the plasma membrane after 30' proNGF treatment or by treatment with Dynasore (an inhibitor for dynamin-dependent internalization). Right: retention on the membrane is sufficient for both wt and mut p75^{NTR} to cause growth cone collapse. ***p<0.001, ns: not significant at the 0.05 level in a one-way ANOVA test with Tuckey's multiple comparisons. Bars are mean±sem. **c-d)** Proposed model for cholesterol-dependent p75^{NTR} signaling on the plasma membrane. **c)** Schematic view of the membrane partitioning of wt p75^{NTR} (green, left) or mut p75^{NTR} mutant (green, right), upon NGF (orange) binding. Cholesterol-rich, signaling-competent regions have increased membrane thickness and contain cholesterol and gangliosides. Partitioning is highlighted by the arrows in opposite directions. **d)** Schematic view of p75^{NTR} signaling at the membrane and downstream internalization. Signaling can occur from cholesterol-poor or cholesterol-rich membrane regions resulting in receptor internalization within clathrin-positive (green) or caveolin-positive (red) endosomes. In our model, interactors of surface-retained p75^{NTR} (magenta), involved in actin-remodeling and growth cone collapse, are more abundant in non-raft regions and indeed this pathway is not impaired by the mutations introduced in mut p75^{NTR}. Conversely, apoptotic signaling effectors (blue) are enriched in raft platforms and therefore efficiently activated only by NGF-bound wt p75^{NTR}. Differently from mature NTs, proNTs at the growth cones cause surface accumulation of p75^{NTR}, which is sufficient for causing growth cone collapse.

We addressed also dynamics and stoichiometry of NGF and proNGF in vesicles moving within neuron axons [3-5,11] (Fig. 3), demonstrating, *e.g.*, that local stall of signaling endosomes containing nerve growth factor (NGF) is responsible for an increased axon elongation for neurons cultured on top of a graphene layer [11]. Moreover, the algorithms and techniques implemented for SPT can be used for analyzing the motion of vesicles imaged with any tool, *e.g.* by confocal fluorescence microscopy [13].

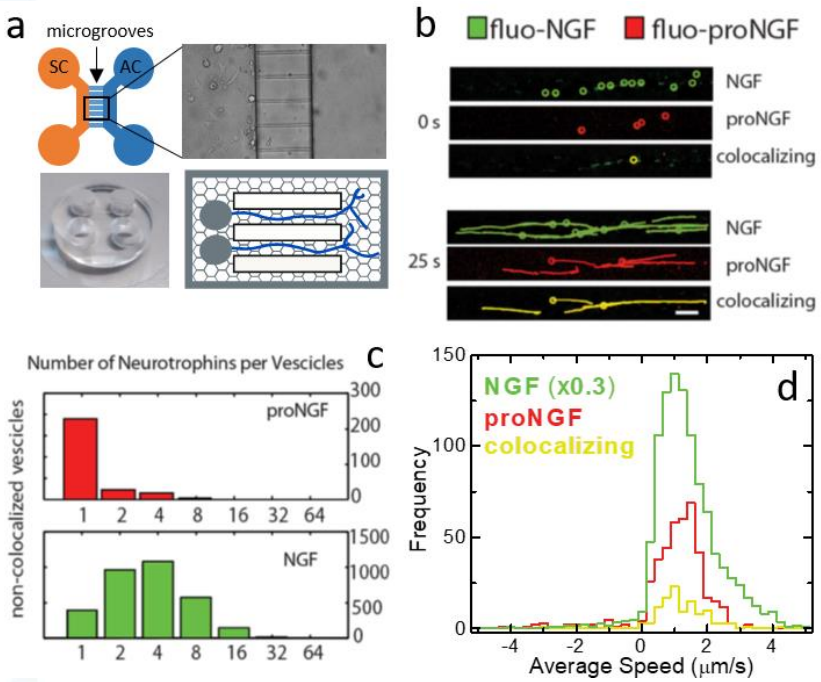

Figure 3. Example of single-vesicle tracking for the transport of fluorescent NGF and proNGF in neuronal axons. **a)** Schemes and images of a microfluidic compartmentalized chamber used to align the axons of neurons grown inside it. **b)** Examples of reconstructed trajectories. **c)** Histograms of the number of fluo-proNGF and fluo-NGF molecules inside moving vesicles. **d)** Example of histogram of average velocities of moving vesicles.

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