High-resolution spatiotemporal analysis of somatostatin receptor type 2 (SSTR2) – Filamin A (FLNA) interaction by single-molecule imaging

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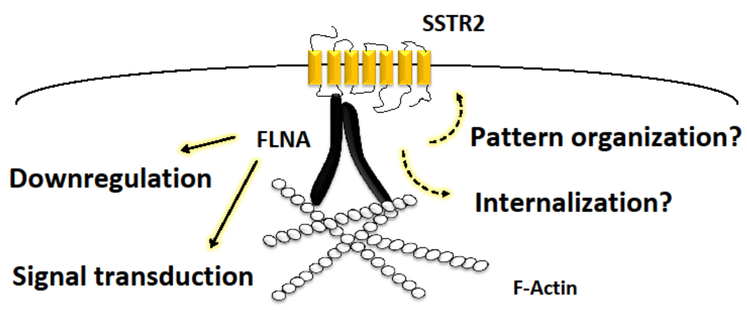
SSTR2 is a G_i coupled receptor used as pharmacological target for GH-secreting pituitary adenomas treatment, however a subset of patients displays resistance to somatostatin analogues. We recently demonstrated that the cytoskeletal protein FLNA plays an essential role in tumor responsiveness by regulating SSTR2 signaling and stabilization after prolonged stimulation in human and rat somatotroph cells.

Aim of this study was to follow the spatiotemporal behavior of SSTR2-FLNA complexes in real time in living cells by high resolution single-molecule imaging.

In particular we wanted to investigate the presence of a spatial distribution of FLNA-SSTR2 complexes at the plasma membrane, to estimate SSTR2 lateral mobility and the involvement of FLNA in regulating this biological phenomenon, and eventually elucidate a possible role of FLNA in SSTR2 clustering organization and internalization after ligand binding.

Crucial role of FLNA in SSTR2 regulation in GH-secreting cells



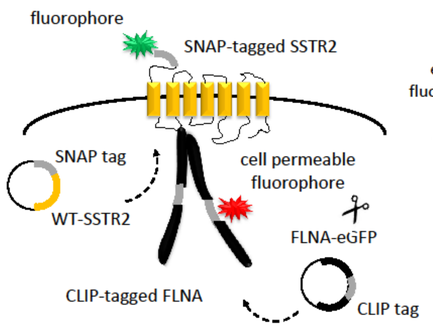
Materials and Methods

- **Cloning:** SNAP-tagged SSTR2 was obtained by fusing a SNAP tag at the N-terminal of a WT-SSTR2 in a pcDNA3 vector; CLIP-tagged FLNA was generated by replacing eGFP with a CLIP tag at the first hinge region of a FLNA-eGFP plasmid.
- **Transient cell transfection:** Transient transfections of CHO, A7, M2 cells were performed using the transfection reagent Lipofectamine2000 according to the instruction of the manufacturer; HEK293AD cells transient transfection (48 hours) have been performed with Effectene.
- **Measurements of cAMP concentration:** Ratiometric FRET experiments to determine cAMP levels were performed in HEK293AD cells co-transfected with SNAP-tagged SSTR2 or WT-SSTR2 together with epac1 sensor.
- **Single-Molecule imaging:** CHO, A7, M2 cells transiently expressing SNAP/CLIP proteins were grown on coverslips at the cell density of 300000cells/w. After 4 hours of transfection cells were labelled with fluorescent dyes (1uM) and imaged at a Nikon TIRF microscope equipped with a EM-CCD camera and Temperature controller. The laser powers were set at 30%.
- **Analysis of receptor mobility:** The distributions of SSTR2 diffusion coefficients were calculated by *u-track* algorithm implemented in *Matlab*.

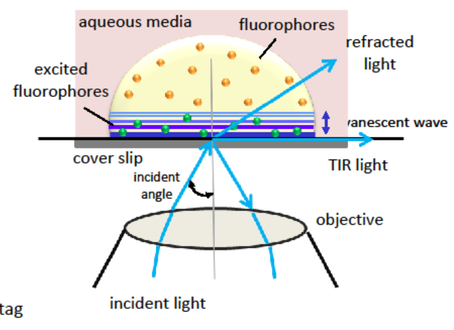
Single-molecule imaging

Single-molecule imaging is a strategy based on labelling SNAP/CLIP-tagged proteins with small organic fluorophores and directly visualize them on the surface of living cells by total internal reflection fluoresence (TIRF) microscopy

SNAP/CLIP-tagged proteins labelled with organic fluorophores



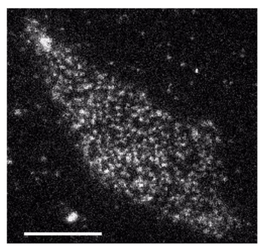
Total internal reflection fluoresence (TIRF) microscopy



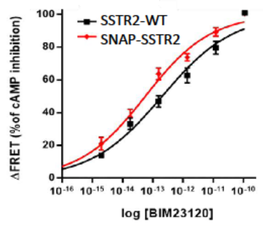
Functional characterization

SNAP-SSTR2

Cell surface localization

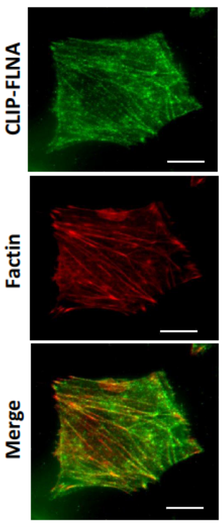


SNAP-SSTR2 cAMP inhibition



CLIP-FLNA

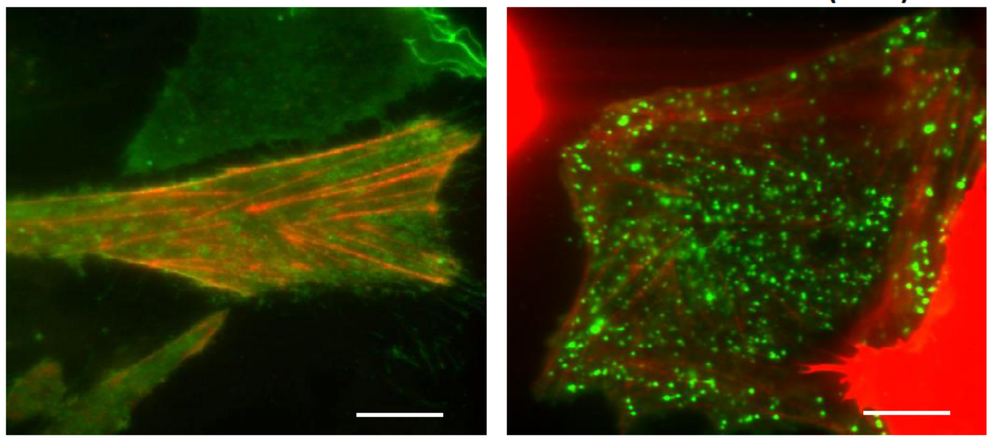
CLIP-FLNA co-localization with actin



Results

FLNA-SSTR2 spatial arrangement at the plasma membrane

Basal **BIM23120 100nM (5min)**

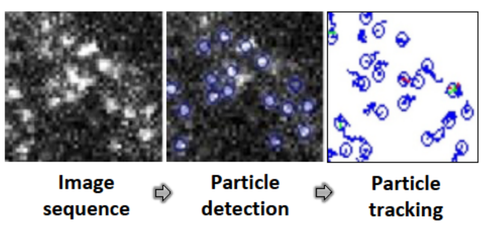


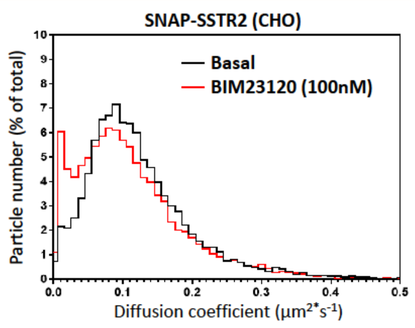
SSTR2 is widely distributed at the cell surface under basal condition whereas upon ligand binding it associates in clusters organized on FLNA fibers

CHO cells overexpressing **FLNA** and SNAP-tagged **SSTR2**

SSTR2 lateral mobility: effect of agonist on receptor speed

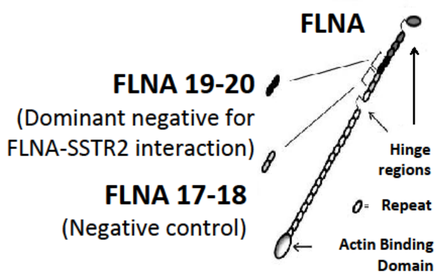
Trajectories of individual SSTR2 particles were used to analyzed their diffusion coefficients

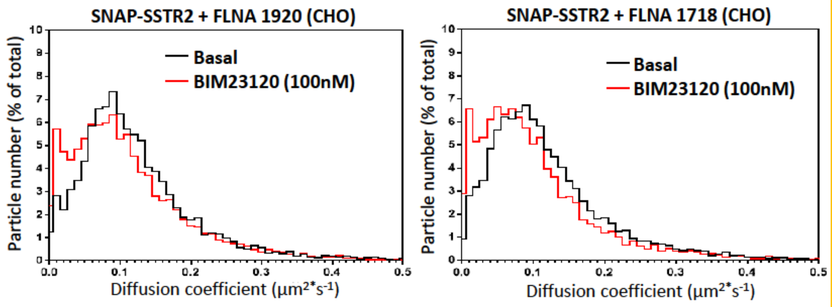




The selective SSTR2 agonist increases the immobile receptors population

…Possible role of FLNA in regulating SSTR2 mobility?



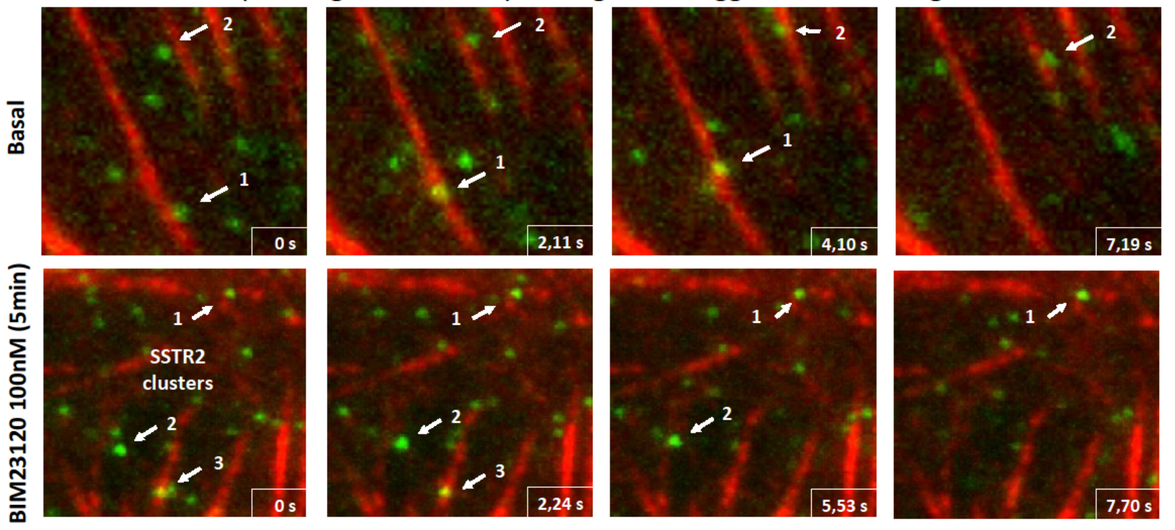


(Confirmed results in human melonama cell lines A7 (FLNA-expressing cells) and M2 (FLNA-deficient cells)

FLNA is not involved in the agonist effect on receptor lateral mobility

Dynamic visualization of SSTR2 – FLNA interactions

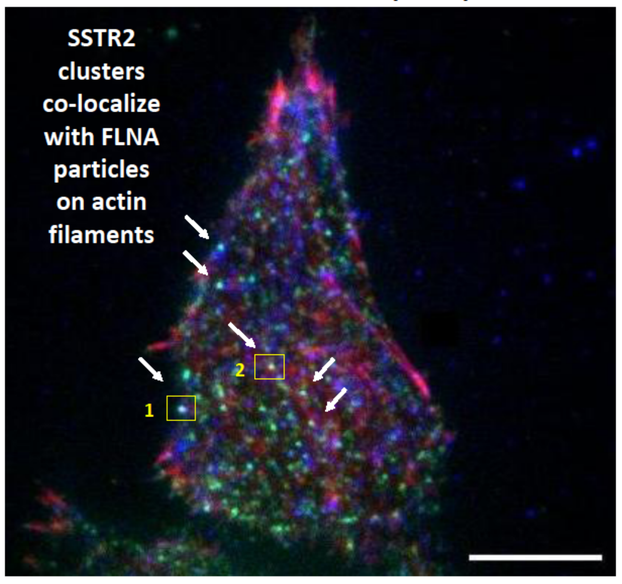
CHO cells overexpressing **FLNA** and expressing SNAP-tagged **SSTR2** at single molecule level

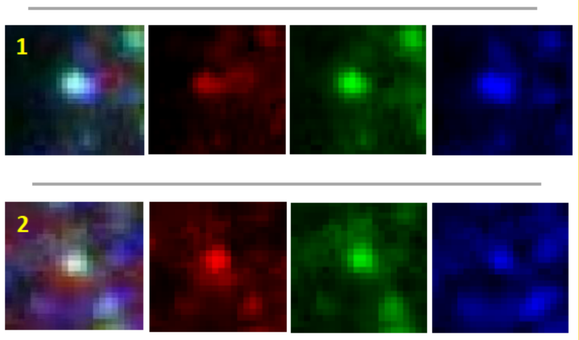


SSTR2-FLNA interactions are extremely dynamic and transient under basal condition, whereas after receptor stimulation static SSTR2s localized along FLNA fibers undergo internalization…

…suggesting a role of FLNA in receptor endocytosis

BIM23120 100nM (5min)





CHO cells co-transfected with **Actin**, CLIP-tagged **FLNA** and SNAP-tagged **SSTR2**; CLIP-tagged FLNA and SNAP-tagged SSTR2 were expressed at single molecule level and labelled with TMR-star and 647 Alexa Fluor BG dye, respectively.

Conclusions

- **Dynamic SSTR2-FLNA interactions become more static and stable upon SSTR2 agonist incubation**
- **The SSTR2 agonist increases the fraction of immobile receptors which co-localize with FLNA**
- **SSTR2 clusters and internalizes along FLNA fibers in stimulated cells suggesting a possible role of FLNA in receptor endocytosis**

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