CHAPTER TWO:

MATERIAL AND METHODS

2.1 Cell culture

Storage of cell line stocks was in the vapour phase of liquid nitrogen, at -190°C in aliquots in culture medium supplemented with 5% (v/v) dimethyl sulfoxide (DMSO). Cells were thawing very fast (<1 min) in a 37°C water bath, and then transferred into 5ml of warm (37ºC) complete media in the 25 cm^2 flask. Cells were left overnight to recover and settle down. The next day the media was changed for fresh one. Established and confluent cells were transferred to the 75 cm^2 flask with the 20 ml complete medium. Cells were cultured at 37°C in the humidified atmosphere containing 5% CO_2.

2.1.1   Human Embryonic Kidney Cell Line, HEK293

Human embryonic kidney cells (HEK293) were originally derived from human kidney cells. This particular line was initiated by the transformation and culturing of normal HEK cells with sheared adenovirus 5 DNA in 1973. HEK293 cells do not have endogenous N-type calcium channels but have endogenous voltage-gated dependent calcium channels current similar kinetic and pharmacological properties to T-type calcium channels[147]. HEK293 cells are commonly used for studying the properties of calcium channels[204,221]. The calcium channels can be easy transfected. The HEK293 cell line was used for experiments to show the difference of N-type calcium channel behaviour and distribution in cells that do not contain the SNARE proteins. HEK293 cells originate from the same precursor cell line as neurons and their basic biological processes are quite similar[222].

Media for HEK293 cell culture:

- Dulbecco's modified Eagle's medium (DMEM), phenol red high glucose (4.5 g/l D-glucose) (Gibco, Paisley, UK)
- 10% Fetal Bovine Serum (FBS) (Gibco, Paisley, UK)
- 1% Glutamax (Gibco, Paisley, UK, 100x)

Passaging of cells was done twice a week, with a ratio 1:5 by removing old media from the 75 cm^2 culture flask, washing the cells with 5 ml of 1x Versene (Gibco, Paisley, UK) and

then adding 4 ml of 1X TrypLE phenol red (Gibco). After 2 min incubation, cells were washed off the flask, resuspended and triturated in fresh media.

2.1.2 Mouse Pituitary Tumour Cell Line, AtT-20/D16-16

The mouse pituitary tumour AtT-20 cell line grow as small round cells in large aggregates. These cells are an excellent model for studying calcium signalling. They express multiple types of high voltage-gated calcium channels but also low voltage-gated calcium channels[148]. The patch clamp method has identified (measurements carried by E. Brown and M. Euston) that the majority of calcium channels present in this cell type are L-type channels. The rest of calcium currents belongs to N-type and P-type calcium channels. The current density of N-type channels in those cells is about 10% [148].

Cells were cultured in media:

- Dulbecco's modified Eagle's medium (DMEM), phenol red high glucose (4.5 g/l D-glucose) (Gibco, Paisley, UK)
- 10% Fetal Bovine Serum (FBS) (Gibco, Paisley, UK)
- 1% Glutamax (Gibco, Paisley, UK, 100X)
- 1% Pen/Strep (Gibco, Paisley, UK, 10000 units/ml, 10000 µg/ml Streptomycin)
- 1% Sodium Pyruvate

Passaging of cells was done twice a week, with a ratio 1:3 by removing old media from the 75 cm^2 culture flask, washing cells with 5 ml of 1x Versene (Gibco, Paisley, UK) and then adding 4 ml of 1X TrypLE phenol red (Gibco). After 2 min incubations, cells were washed off the flask, resuspended and triturated in fresh media.

2.1.3 Rat Adrenal Pheochromocytoma Cell Line, PC12

The rat adrenal pheochromocytoma (PC12) cell line was originally derived from a rat adrenal medulla tumour in 1976 by Green and Tischler[149]. They are a combination of neuroblastic cells and eosinophilic cells with an embryonic origin. The PC12 cell line

grows as round or polygonal shape cells in small groups[149]. Under established conditions, PC12 cells look like chromaffin cells. Upon treatment with nerve growth factor (NGF) cells stop growing, expand and become neuron-like cells[149]. PC12 cells are a good model for studying secretion[223]. Despite the expression of SNARE and other exocytosis proteins[224], PC12 express L-type calcium channels as undifferentiated cells. After differentiation with NGF, they also start to express also N-type calcium channels[150].

Cells were cultured in media:

- RPMI, phenol red, enhanced with non-essential amino acids and sodium pyruvate (Gibco, Paisley, UK)
- 10% Horse serum (Hyclone, Buckinghamshire, UK)
- 5% Fetal Calf Serum (Hyclone, Buckinghamshire, UK)
- 1% Glutamax (Gibco, Paisley, UK, 100X)
- 0.1% Gentamicin (Gibco, Paisley, UK, 50 mg/ml)
- 1% Sodium Pyruvate

Passaging of cells was done every 4-5 days, with a ratio 1:3 by removing old media from the 75 cm^2 culture flask, washing the cells with 5ml of 1x Versene (Gibco, Paisley, UK) and then adding 4 ml of 1X TrypLE phenol red (Gibco). After 2 min incubations, cells were washed off the flask, resuspended in 4ml of fresh media, transferred into 15 ml conical falcon and spun down in a centrifuge for 3 min at about 500 g. The solution was decanted, cells pellet was re-suspended in 8 ml of fresh media and transferred into a fresh collagen coated flask.

Differentiation NGF medium (50 ml):
- 48.5 ml RPMI culture media
- 100 ng/ml NGF β (Sigma-Aldrich H9666-10UG)
- 1% Horse serum (Hyloclone)
- 1% Penicillin Streptomycin

2.2 Molecular biology

The genetic constructs used for the experiments were generated as a part of previous research projects and are regularly used by member of the laboratory.

Construct name	Source	Antibiotic resistance
R-GECO-C1-SNAP25	Cloned by K. Hiersemenzel	Kanamycin
EGFP-C1- CaV2.2-α_{1B}	Cloned by K. Hiersemenzel	Kanamycin
EGFP-C1- CaV2.2-α_{1B} [Δ18a]	Cloned by K. Hiersemenzel	Kanamycin
PAmCherry-C1-CaV2.2-α_{1B}	Cloned by K. Hiersemenzel	Kanamycin
PAmCherry-C1-CaV2.2-α_{1B} [Δ18a]	Cloned by K. Hiersemenzel	Kanamycin
Cavb3	Addgene # 26574	Ampicillin
CaVa2d1	Addgene #26575	Ampicillin
pEGFP-N3	Clontech	Kanamycin
pEGFP-N3 co-expressed with pmCherry-N1	Clontech	Kanamycin
EGFP fused to mCherry by a 12 amino acid linker on a pCDNA3.1 backbone	Cloned by D.Kavanagh	Kanamycin

Table 2.1 List of constructs used in this thesis.

2.2.1 Plasmid maxi prep preparation

For the plasmid DNA purification from bacteria, the maxi prep kit was used:

1. Dip the pipette tip in plasmid solution and add to 40 µl XL10-Gold Ultracompetent cells (Agilent Technologies, Stockport, UK)

2. Incubate on ice for 10 min

3. Heat shock at 42°C for 30sec (water bath)

4. Incubate on ice for 5 min

5. Add 500 µl LB

6. Incubate at 37°C, shaking 200 rpm for 30 min

7. Add 10 µl to 1st plate and 100 µl to 2nd plate

8. Incubate plates overnight at 37°C, to grow colonies

For plasmid preparation, single colonies were picked from plates and cultured in 5 ml LB tubes with the correct antibiotic (ampicillin or kanamycin). The tubes were incubated for about 5 h at 37°C on a shaker. After that time cultures were transferred into 250 ml LB flask and incubated for 16 h at 37°C on a shaker before DNA extraction using the HiPure Plasmid Maxiprep Kit (Invitrogen), following the instructions.

Harvesting of bacterial culture

- Centrifuge bacterial cultures (3000 x g, 4°C) for 30 min
- Decant supernatant and remove all remaining media

Using PureLink HiPure Plasmid DNA Purification Kit (Invitrogen, Paisley, UK)

1. Equilibrate. Apply 30 ml Equilibration Buffer to the Column via funnel lined with fluted filter paper. Allow the solution in the column to drain by gravity flow

2. Resuspend. Add 10 ml Resuspension Buffer with RNase A to the cell pellet and resuspend the pellet until it is homogeneous

3. Lyse. Add 10 ml Lysis Buffer. Mix gently by inverting the capped tube five times. Incubate the tube at RT for 5 min.

4. Precipitate. Add 10 ml Precipitation Buffer. Mix immediately by inverting the tube until the mixture is homogeneous.

5. Bind. Load the mixture onto the equilibrate column via funnel lined with fluted filter paper to collect precipitate. Allow the solution in the column to drain by gravity flow

6. Wash. Remove funnel and filter paper and add 60 ml wash Buffer to the column. Discard the flow-through after Wash Buffer drains from the column.

7. Elute and precipitate. Place the sterile 50 ml centrifuge tube under the column containing 10.5 ml 70% isopropanol. Add 15 ml Elution Buffer to the column Allow the solution to drain by gravity flow. The elution tube contains the purified DNA. Mix well.

8. Wash. Centrifuge the tube at 10 000 g for 30 min 4°C. Remove and discard the supernatant.

9. Wash the DNA pellet in 5 ml 70% ethanol. Centrifuge the tube at 12 000 x g for 5 min at 4°C. Remove the supernatant.

10. Resuspend. Air-dry the pellet in incubator than resuspend the purified plasmid DNA in 250-500 ul Milli-Q water. To determine nucleic acid concentration nanodrop was used.

2.3 Sample preparation

2.3.1 Coverslips preparation and cells counting

Cells for each experiment were seeded onto coverslips prepared due the protocol.

- Submerge coverslips in to 0.1 M NaOH, 0.1% Decon-90 for 30 seconds with constant sonication in a sonicating water bath.
- Wash by submerging sequentially in 3 x 1L of deionised water.
- Submerge in 100% ethanol.
- Submerge in 100% acetone.
- Wash in 1L of deionised water to remove acetone.
- Place directly in to a dish containing 100 mg/ml poly-D-lysine (Sigma, P6407).
- Incubate with gentle agitation for 1 hour.
- Remove coverslips individually, rinse in deionised water and air dry in a laminar flow hood.
- UV irradiate coverslips to sterilise.

Coverslips for PC12 cells were additionally coated with 1 mg/ml collagen from a human placenta, Bornstein and Traub type IV (Sigma, C5533) overnight.

Each time the total number of cells were counted by using haemocytometer. The gridded area of haemocytometer consists of nine 4x4 squares with 0.1 mm^3 volume. The number of cells in 1 ml can be calculated by;

$$x = \frac{number\ of\ cells\ in\ 4x4\ square\ x\ 1000\ \mu l}{0.1\ \mu l}$$

According to the different growth rate of each cell line, they were plated in different densities to reach 80% confluency for transfection. For HEK293 cells 0.6 million cells, for AtT-20/D16-16 0.8 million cells and for PC12 1.6 million cells were plated per well.

2.3.2 Cell transfection

The introduction of nucleic acids into eukaryotic cells by non-viral methods is defined as transfection. It is a powerful method which gives the opportunity to study protein expression in the cell. Cloned genes can be transfected into cells for biochemical characterization, investigation of the effects of gene expression on cell growth or to produce a specific protein.

To express the human brain N-type voltage-dependent calcium channels in HEK293 and PC12 cells, triple transfection was performed, according to the protocol:

- Remove the old media from the 75 cm^2 culture flask
- Rinse cells by 5 ml Versene (Gibco, Paisley, UK)
- Add 4 ml of Trypsin-EDTA (Gibco, Paisley, UK)
- After 2 min incubation of cells, wash off the flask with phenol-red-free (with serum) DMEM, and pippete cells
- Count cells in haemocytometer, there should be ~40 cells per quadrant, then add 1ml of resuspended cell to each well + 1ml phenol-red-free (with serum) DMEM
- Leave for the 24 h

For transfection was used serum free, phenol-red free DMEM medium:

- Mix in one centrifuge tube three x 2 μl (concentration 1 μg/μl) N-type subunits plasmids (EGFP-α1B subunit / PACherry-α1B for PALM technique, β-subunit and α2δ-subunit) with 250 μl serum free, phenol-red free DMEM (per 1 well)
- In the second centrifuge tube mix 9 μl Lipofectamine2000 (Invitrogen, Paisley, UK) with 250 μl serum free, phenol-red free DMEM (per 1 well)
- Leave for 15 min in room temperature
- Mix the first and second centrifuge tubes and leave for another 15 min in room temperature
- Replace old medium in wells with fresh phenol-red free (with serum) DMEM
- Add transfection mix into wells (500 μl per well) in a dropwise manner
- Incubate cells in 37°C with 5% CO_2 for 48h

For single plasmid transfection, 4 μl of 1 μg/μl DNA with 200 μl serum free, phenol-red DMEM and 6 μl Lipofectamine2000 (Invitrogen, Paisley, UK) with 250 μl serum free, phenol-red free DMEM was used per 1 well.

2.3.3 Cell fixation

Cell fixation immobilises cellular components and makes it possible to permeabilise a cell membrane to allow antibodies to access intracellular structures. In this thesis, Paraformaldehyde 4% (w/v) was used for 20 min to fix cells. After washing 2x with PBS cells were incubated with 50 mM NH_4Cl for 4 min to quench autofluorescence.

2.3.4 Immunostaining

Immunostaining is a method to detect specific endogenous proteins in the sample. In this method, a protein-specific primary antibody is used and fluorescent labelled secondary antibody. The sample was first fixed due to the protocol described above (2.3.3) followed by:
- Wash cells 2x with PBS.
- Incubate with 0.5% triton X100 (a detergent to solubilise the membranes) diluted in PBS for 4 min.
- Wash cells 2x with PBS.

- Incubate with PBS + 0.5% Fish Skin Gelatine (FSG) for 15min (a non-specific protein acts as a blocker for spurious antibody binding).

- Wash cells 2x with PBS.

- Dilute the antibody (1:1000) in PBS +0.5% FSG, mix and centrifuge. Incubate cells at RT for 3 h (or in 4°C overnight).

- Wash thoroughly at least 3x in PBS+0.5% FSG.

- Add 1:1000 dilution of secondary antibody. Incubate for only 1h.

- Wash extensively 3x with PBS.

2.3.5 Unroofing method

The inner surface of the cell membrane can give us a lot of information about structures in cells. As the thickness of the whole attached cell is about 10 μm, it is an obstacle to see only proteins at the membrane. That is why plasma membrane sheets are ideal for the microscopic study of membrane proteins. Exposing plasma membrane to high-power ultrasonic waves was the method used to visualise the cytoplasmic surface with expressed N-type calcium channels in HEK293 cells and to check whether Cav2.2 are in clusters or randomly distributed. Sheets were made by using ultrasonic waves (figure 2.1) to remove the upper cell membrane and cytoplasmic components and retain the bottom one (membrane attached to coverslip)[151]. The experiment was conducted at room temperature in Sonication buffer.
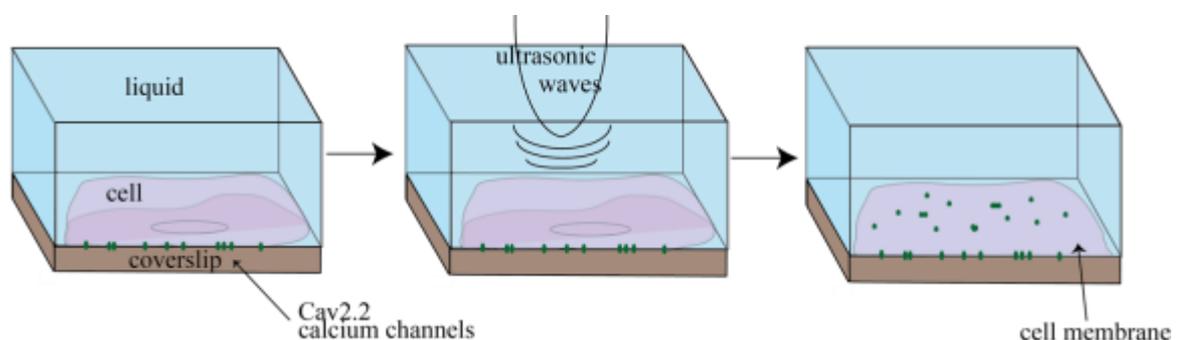

Figure 2.1. Unroofing protocol. Transfected cells with EGF-Cav2.2 calcium channels were exposed for low-power sonication waves to remove the upper cell membrane and visualise the bottom one with expressed N-type calcium channels.

Sonication buffer, in 500 ml MiliQ water, was made fresh:

- 120 mM Potassium glutamate (12,2 g)
- 20 mM Potassium Acetate (1g)
- 10 mM EGTA (2 g)
- 0.5 mM DTT (aliquots 500 µl in freezer)
- 0mM HEPES (2.4 g)

The solution was adjusted to pH7.2 using NaOH. Cells were exposed to 5 sec ultrasonic burst from 40% of maximum power in stabilisation buffer, then fixed in PFA 4% immediately after 'unroofing'.

2.3.6 Mounting samples in Mowiol

Samples on coverslips were mounted in aqueous mounting media – Mowiol 4.88 (Sigma-Aldrich, UK). This mounting medium is liquid when the sample is mounted and becomes solid after few hours. Mowiol 4.88 has the same refraction index as immersion oil accordingly is working well with the oil immersion objective lens. A drop of Mowiol 4.88 was placed onto the microscope slide and coverslip was put on top. Samples were left at room temperature overnight in the dark to dry and then stored in 4°C. The next day CoverGrip (Biotium) was added to the edges of the coverslip to seal the sample. The stock was prepared by mixing 2.4 g of Mowiol 4.88, 6 g of glycerol and 6 ml dH_2O and left for 2 h stirring at room temperature. 0.2 M Tris (pH8.5) was added (12 ml) and heated to 50°C for 10 min with stirring. When dissolved, Mowiol was transferred to a Falcon tube and centrifuge at 5,000 xg for 15 min to remove any non-solubilised particles. Aliquots were stored in the freezer at -20°C.

2.3.7 Preparation of bead samples

For calibration of the STED microscope, FluoSpheres™ - Carboxylate-Modified Microspheres, 0.02 µm, yellow-green fluorescent (ThermoFisher Scientific, #F8787) microspheres were used. Beads were sonicated for 10 minutes in the ultrasonic bath. Aggregates were pelleted by centrifugation and the supernatant was diluted in 100 mM $NaHCO_3$ (in ddH_2O). Serial dilution from 10,000 to 100,000 was prepared and sonicated

again for 10 min. Bead particles were attached to the coverslips coated with Corning™ Cell-Tak Cell and Tissue Adhesive (FisherScientific, #10317081). Cell-Tak is a specifically-formulated protein solution extracted from *Mytilus edulis* (marine mussel). Mussels produce these proteins to anchor themselves to solid surfaces[152]. Cell-Tak (1.5 μl) was added to the 20 μl of 100 mM $NaHCO_3$ in ddH_2O and vortexed immediately. The mix of 20 μl was added to the centre of cleaned coverslip (2.3.1) and left for 20 minutes. After this time, the liquid was removed carefully and a sticky spot was left. Diluted fluorescent microspheres were added to the coverslip as soon as possible, and left for at least 20 minutes and then removed. Coverslips were mounted in Mowiol 4.88 and kept in the fridge 4°C.

2.4 Microscopy methods

2.4.1 Refractive index and Nyquist sampling rate

Spherical aberration occurs when incoming light refracts after passing through a spherical surface. This results in lower resolution. This phenomenon can be minimised by using the right lens together with its specific coverslip thickness (usually 0.15 mm) and appropriate refractive index medium. Refractive index is the ratio of the speed of the light in a vacuum to that in the second medium of higher density[101].

In the image processing context, the sampling rate is the number of recorded pixels or voxels in a digital image per unit distance[153]. This parameter decides on the number of pixels in the digital image. The large the image size the smallest sample rate. The Nyquist-Shannon theorem says to obtain the maximum resolution, the sampling rate should be greater than double the bandwidth of the microscope's PSF[101,154]. For the Widefield microscope, the Nyquist sampling rate is given by:

$$\Delta_x = \frac{\lambda_{em}}{4n\sin(\alpha)} \qquad [2.1]$$

where: λ_{em} is the emission wavelength and n is the lens refractive index. For the confocal microscope, the sampling rate must be divided by 2;

$$\Delta_x = \frac{\lambda_{ex}}{8n\sin(\alpha)} \qquad [2.2]$$

where: λ_{ex} is the excitation wavelength[154]. At confocal microscope, the pixel size can by changed by scanning at a higher image resolution and higher zoom[101]. For z-stacks, it is important to use sample rate 2.3 times smaller than the z-resolution of the objective[101].

2.5 Imaging buffers

The standard buffers were prepared due to the protocols in table 2.2.

Buffer	Preparation
PBS	PBS tablet was dissolved in 200 ml of ddH$_2$O to obtain 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution (pH= 7.4).
Ringer Buffer	For 400ml buffer: 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM D-(+)-Glucose, 20 mM HEPES pH=7.2-7.4, Osmolarity 290-310 mOsm
STORM Buffer (MEA)	50 mM Cystamine in PBS, 0.5 mg/ml glucose oxidase, 40 μg/ml catalase, 10% (w/v) glucose. Stock stored at -20°C
Internal solution for whole cell patch clamp	For 100 ml solution in MiliQ water: 100 mM D-(+)-Glucose, 1 mM CaCl$_2$, 120 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES, 4 mM ATP, 11 mM EGTA pH=7.2, Osmolarity 290-310 mOsm

Table 2.2 Buffers for imaging

2.6 Microscopy methods and image processing

2.6.1 Confocal Laser Scanning Microscopy

Confocal microscopy imaging was achieved by using Leica SP5 single molecule detection (SMD) confocal laser scanning microscope (CLSM) using a 63x 1.4 NA HC Apo oil immersion objective lens and a pinhole set to the 1 Airy unit. EGFP probes were excited by 488 nm CW Argon gas laser. mCherry and Alexa Fluor568 were excited by 561 nm CW Helium-Neon gas laser. The acousto-optical beam-splitter (AOBS) was used instead the dichroic mirror to separate and detect selected wavelengths[101]. EGFP emission was collected between 500-550 nm and both mCherry and Alexa Fluor568 emission were collected between 600-670 nm. To the notch filter was used to selectively eliminate the 488 nm or 561 nm excitation wavelength while passing on another wavelength. A photomultiplier tube (PMT) set to a gain 600-800 V was used to detect emission. The pixel size was about 40 nm. 3D images were obtained by using 1.4 NA x 63 immersion oil and images were set to contain 1024x1024 pixels. Z-steps were set to 130 nm as required for Nyquist sampling rate[154].

2.6.2 Stimulated emission depletion microscopy

Continuous-wave gated stimulated emission depletion (CW-gSTED) imaging was achieved by using Leica SP5 SMD CLSM with a 100x 1.4 NA HC Apo oil immersion objective lens. EGFP probes were excited by 488 nm white light laser (WLL) pulsing at 80 MHz. The CW 592 nm was used as a depletion laser. Emission was collected as for CLSM and detected with a time-gated Leica HyD hybrid detector. Time-gated detection from 0.5-6.50 ns was used to deplete outer PSF fluorescence before the detection. Sequential scanning was used to obtain confocal and gSTED images. The pixel size was about 20nm. For calibration of gSTED resolution, fluorescent beads (section 2.3.7) were used (figure 2.2).
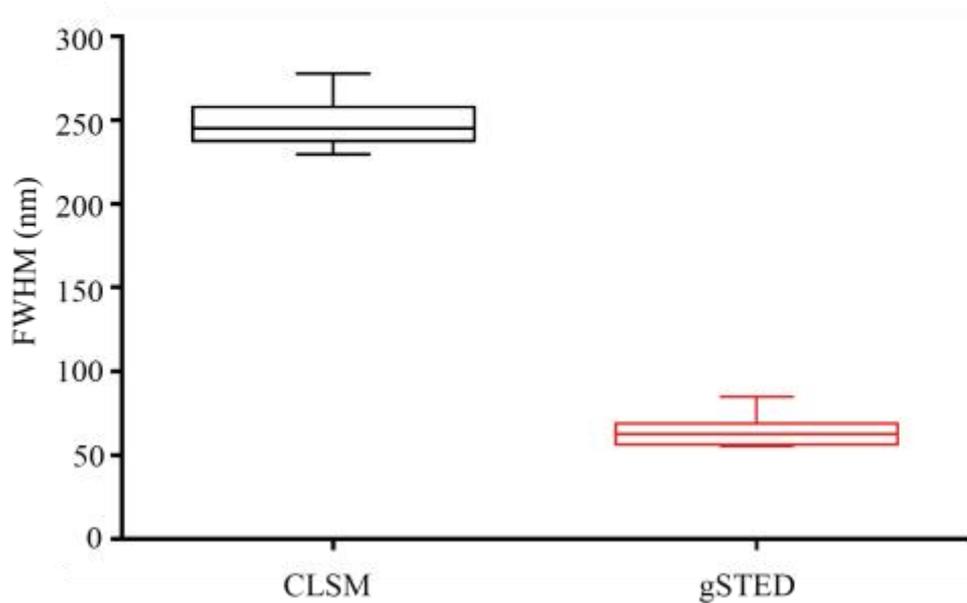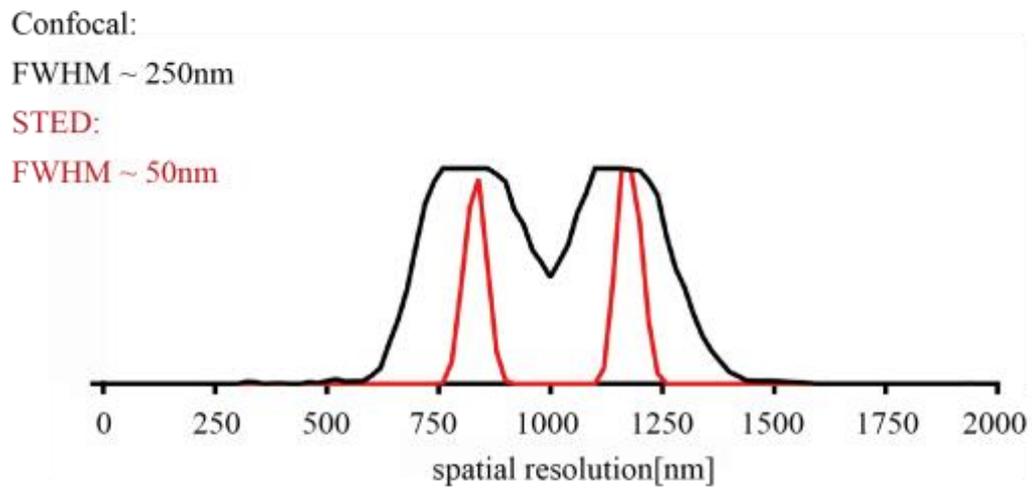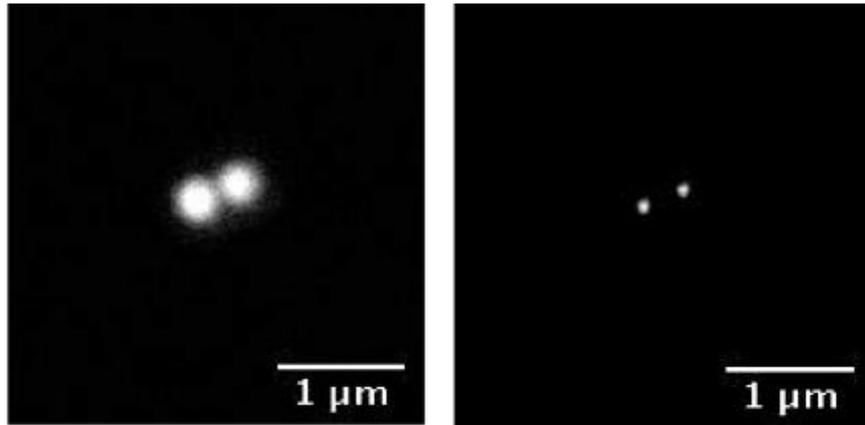
Figure 2.2 Confocal and gSTED images of ~20 nm fluorescent beads to determine resolution. Beads were excited by 505 nm laser. Box plots for the fitted FWHM of the beads indicate increased resolution obtained with gSTED over confocal (CLSM) imaging.

2.6.3 Fluorescence lifetime imaging microscopy

TCSPC FLIM-FRET imaging was achieved by using the Leica SP5 SMD CLSM microscope included Picoharp 300 TCSP module and single photon avalanche diode (SPAD) detectors for fluorescence lifetime imaging. Images were acquired with 32-bit SymPhoTime v5.4.4 (PicoQuant) software with maximum photon count rate of 10^5 photon counts per second. A 63x 1.4 NA HC Apo oil immersion objective lens was used and a pinhole set to the 1 Airy unit. To excite the donor fluorophore EGFP, the 488 nm WLL was used with a pulse frequency 40MHz. As an acceptor, endogenous proteins immunolabelled (2.3.4) were used, and as a secondary antibody Alexa Fluor568-conjugated anti-IgG (Invitrogen). The images were set to a 128x128 pixel format. The pixel size was 322 nm. Analysis of FLIM was done by using SymPhoTime v5.4.4 (PicoQuant). Fluorescence decays curves were fitted to a bi-exponential decay model. Fluorescence lifetime was estimated by using a pixel-by-pixel fitting. To determine the pixel FRET efficiencies the Microsoft Excel VBA script designed by R. Saleeb. The single-pixel amplitude-weighted average fluorescence lifetime (τ_{amp}) was calculated using the equation; $\tau_{amp} = (A_1\tau_1 + A_2\tau_2) / (A_1 + A_2)$, where A_1 and A_2 are the amplitudes of the decay components and τ_1 and τ_2 are the fluorescence lifetimes. The FRET efficiency was calculated using the equation; $E_{FRET} = (1-\tau_{DA}) / \tau_D$, where τ_{DA} is the lifetime of the donor (EGFP) in the presence of the acceptor (Alexa568) and τ_D is the single pixel median amplitude weight fluorescence lifetime of the EGFP donor-only sample.

2.6.4 Widefield and Total internal reflection microscopy

Widefield and TIRF imaging was carried out using an Olympus IX-81 TIRF+ microscope with a 150X 1.4 NA UAPON immersion oil lens. An EMCCD camera (Hamamatsu, UK) was used for detection of emission, which has a pixel array of a 512 x 512, 16 μm pixels, resulting in pixel size in the image of 106 nm. As excitation sources, 100 mW laser diodes producing 405 nm, 491 nm, 561 nm and 640 nm wavelength excitation beams were used. The TIRF angle setting was adjusted through the Olympus software Xcellence, setting the correct angle for each laser excitation beam individually.

2.6.5 Photoactivated Localization Microscopy

For imaging EGFP 491 nm illumination laser in TIRF mode was used. For Photoactivatable mCherry (PAmCherry) single molecule imaging acquisition was done setting multiple cycles of activation with 405 nm laser (31 ms) followed by imaging with 561 nm laser (61 ms frame rate), both in a TIRF mode. Emitted fluorescence was detected using the EMCCD camera with an EM Gain of 100-400. This cycle of activation and acquisition was repeated between 100 and 300 times with the activation pulse duration increasing gradually during the experiment. The fixed cells were imaged in PBS in room temperature and live cells were imaged in warm (37°C) Ringer Buffer at room temperature. The nose-piece stage (Olympus) was used to minimise stage drift[75].

2.6.6 Stochastic Optical Reconstruction Microscopy

All dSTORM experiments were performed in TIRF mode using an Olympus IX-81 (2.5.3). For imaging EGFP 491 nm illumination laser in TIRF mode was used. For dSTORM, endogenous proteins were immunolabelled (2.3.4) and as a secondary antibody, Alexa Fluor647-conjugated anti-IgG (Invitrogen) was used. Samples were prepared by adding 250 μl of the MEA buffer (Table 2.2) onto the hollowed slide and placed coverslip (cells down) onto the liquid. The coverslips were sealed with picodent twinsil® 22 (Picodent, DE) and left in the fridge for 30min until hard. For activation Alexa647 100% 640 nm excitation laser was used followed by 4 sets of 3000 frames cycle. Adding the 405 nm laser excitation pulse between each set, allowed to activate more molecules over time. The frame rate was 50 ms, and EM Gain was constant equal 300.

2.6.7 Abberior Cage552-ω-conotoxin for single molecule localization

To detect the position of endogenous Cav2.2 calcium channel the ω-conotoxin conjunct with Cage552 fluorescent dye was used. Almac group (Craigavon, UK) made Abberior Cage552-conotoxin in ratio 1 molecule to 1 molecule with molecular weight 3829.5, and 17 amino acid size. One molecule of Cage552 was bound to one molecule of ω-conotoxin GVIA through unmasking the thiol moiety on the N-terminus. The final product was isolated using reversed phase High-performance liquid chromatography (HPLC) method. This method is a faster form of column chromatography. The product was analysed by electrospray ionisation mass spectrometry (ESI-MS). The molecular peak from ESI-MS

corresponds to the total molecular mass of the labelled protein. Abberior Cage552 is a non-fluorescent substance which, upon photolysis at 360−440 nm, readily transforms into a deeply red and highly fluorescent dye. It absorbs around 552 nm and emits around 575 nm[155]. That dye can be used for STORM and PALM technique. The advantage of conotoxin conjugated to Cage dye is that the fluorescence signal from the molecule is observed only once because after a few seconds it is bleached forever. Using this tool one single N-type channel can be recorded. The comparison between unlabelled ω-conotoxin and Cage552-ω-conotoxin was made by using the patch clamp technique by M. Euston and E. Brown (Heriot-Watt University). To block 50% of N-type calcium channels 0.2 ± 0.03 μM of conotoxin must be used whereas for tagged conotoxin it is 1.7 ± 0.34 μM (figure 2.3). The protocol for incubation cells with Cage552-conotoxin was modified a few times. The final protocol was carried out in the dark, with following steps:

- Transfected cells were incubated with Ringer Buffer with mix of 1% Bovine Serum Albumin (BSA) / Fish Skin Gelatine (FSG) solution for about 1 h. BSA and FSG are blocking buffers that minimize non-specific binding of molecules. Buffer was filtered and photobleached.
- 1 μl of 2 μM Tetrodotoxin (TTX) was added to cells for 1min, to block Na^+ channels
- 1 μl of 0.5 mM Abberior Cage-conotoxin was added to cells for 5 min, to block N-type calcium channels (final concentration 0.5 μM)
- Cells were washed 6 times with Ringer Buffer mix with a 1% BSA/FSG solution

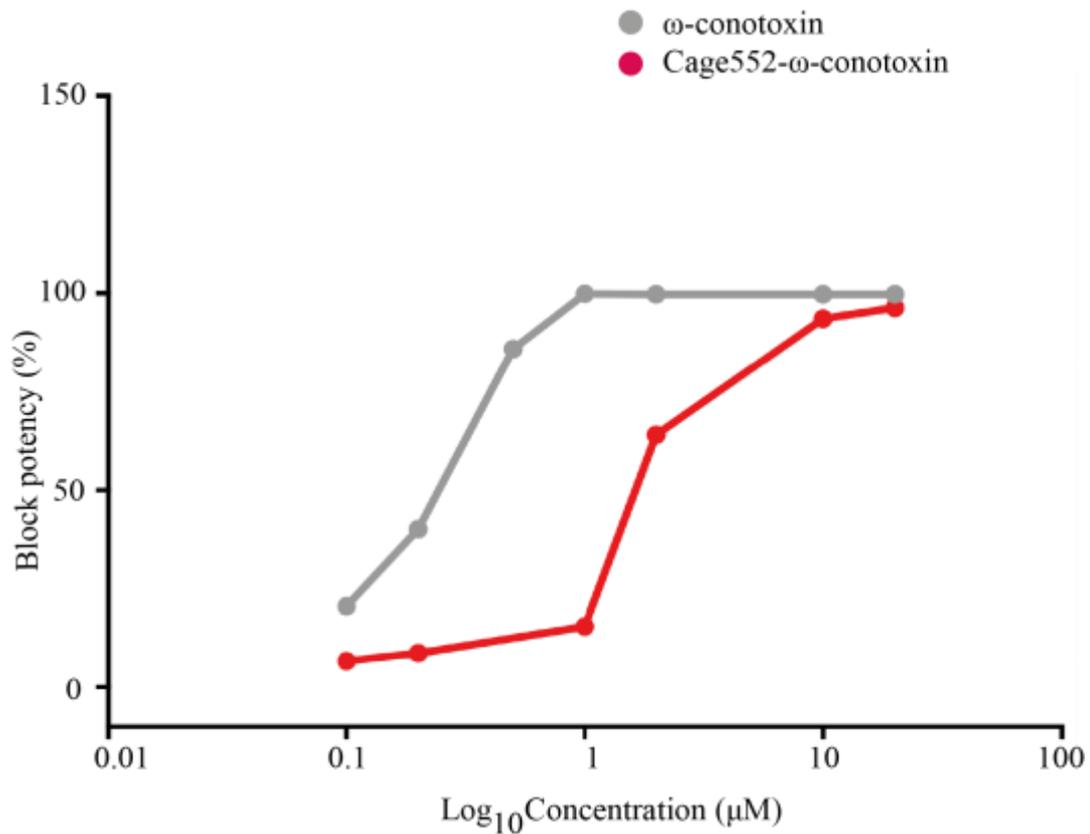Cell were imaged using PALM technique described above (2.5.5).

Figure 2.3 The comparison between unlabelled ω-conotoxin and Cage552-ω-conotoxin. To block 50% of N-type calcium channels $0.2 \pm 0.03\mu M$ of conotoxin must be used whereas for tagged conotoxin it is 1.7 ± 0.34 μM.

2.7 Microscopy images processing

2.7.1 Image deconvolution

Deconvolution is a process that improves the contrast and resolution of images captured in the microscope. The structure is based upon a suite of methods that are designed to remove or reverse the blurring present in microscope images induced by the limited aperture of the objective. The 3D CLSM and widefield images were deconvolved with Huygens Professional (Scientific Volume Imaging) software. The quality threshold was changed to 0.01, and SNR was changed depends on data noise.

2.7.2 Colocalization analysis

To analyse colocalization, the Fiji plugin Coloc 2 was used. To analyse the images for this thesis, the Manders correlation coefficient (MCC) was chosen. MCC measures the proportion of the one channel signal (M1) coexistent with a signal of the second channel (M2)[156]. The values range from 0 to 1. If the two channels colocalized, the value is close to 1. This coefficient is well-suited for analysis even when the intensity signals from two proteins strongly differ[156]. The correlation coefficient can be determined by equation for each channel:

$$M1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i}$$

$$[3.1]$$

$$M2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$$

Where S1 represents the signal intensity of pixels in the channel 1, S2 represent the signal intensity of pixels in the channel 2, $S1_{i, coloc} = S1_i$ if $S2_i > 0$ and $S2_{i, coloc} = S2_i$ if $S1_i > 0$ [156].

2.7.3   Single molecule localisation analysis

Data acquired by PALM or dSTORM were analysed by using the Fiji plugin ThunderSTORM[157]. This plugin detects and localises single molecules by distinguishing the PSF in the PALM or dSTORM images and determine the coordinates of each localised molecule (more details in 1.4.6). As a result, we get rendered images.

2.7.4   Spatial organisation analysis

The spatial distribution of individual molecules was analysed by using coordinates information from PALM or dSTORM ThunderSTORM results. To check if molecules form

clusters, Ripley's K function was used with its transforming L-function. Analysis was performed using custom written Matlab algorithm. This function describes the points distribution pattern in a specified area of interest by taking into account all neighbours[158]. Deviations from 0 indicate that molecules are in clusters or dispersed (figure 2.4). Although the analysis with Ripley's K function gives information about overall distribution, it does not provide information about cluster size, cluster position, the number of clusters and number of localisation per cluster. It is also required a high density of localisations to recognise the cluster pattern from random one. These problems can be solved by using Bayesian cluster analysis[159]. This method is based on Ripley's K function and check cluster distribution for each radius and threshold and determine the most likely scenario. Bayesian cluster analysis was run in RStudio Server software (USA).

To check the N-type calcium channels organisation relative to the tSNARE proteins, the nearest neighbour Matlab (The Mathworks, Inc.; Natick, MA) script written by C. Rickman (Heriot-Watt University) was used. The number of detected molecules within given distance from each centre of diffraction limited TRIFM coordinates to the centre of dSTORM coordinates was measured.
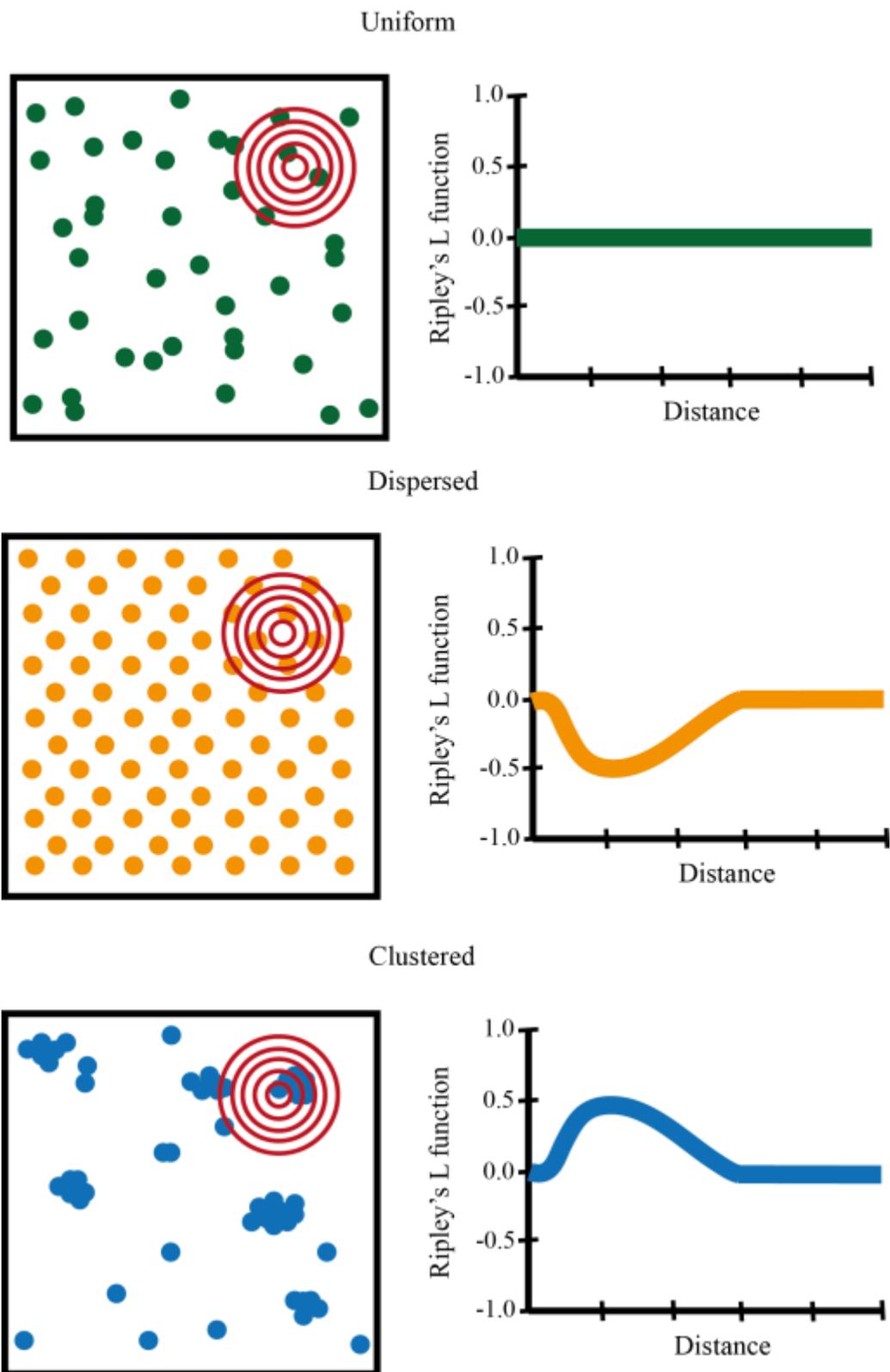
Uniform

Dispersed

Clustered

Figure 2.4 The spatial distribution of individual molecules analysed with Ripley's K function. Deviations from 0 indicate that molecules are in clusters or dispersed.

2.7.5 Tracking of single molecules

The mobility of N-type calcium channels was analysed with high-precision tracking of thousands of molecules by using single-particle tracking PALM (sptPALM).

The mobility of proteins in the membrane can be estimated by the diffusion coefficient. The diffusion coefficient is the constant ratio between the molar flux as a result of the molecular diffusion and the gradient in the concertation of the species[160]. The diffusion coefficient (D) depends on the size and shape of a molecule, interaction with solvent and also viscosity of solvent[161] and it is based on the Stokes-Einstein equation

$$D = \frac{kT}{6\pi r\eta} \qquad [2.3]$$

where: k is Boltzmann constant, T is absolute temperature, r is the radius of the spherical molecule and η is viscosity[162,160]. The Stokes-Einstein equation is derived by Einstein in his PhD thesis and describes the diffusion coefficient of a particle undergoing Brownian Motion in an inactive fluid at uniform temperature Brownian motion describes the continuous but random motion of a molecule suspended in a liquid[163]. In recent research, it was presented that membrane proteins like SNARE are relatively immobile[73,164] and membrane domains containing syntaxin-1 and SNAP-25 in the plasma membrane of PC12 cells are rather static or move with very low speed[164,165]. Taking as an example a free syntaxin-1 (33kDa) which diffusion coefficient is around ~0.1 $\mu m^2 s^{-1}$ [165] or phosphatidylcholine (0.8kDa) which diffusion coefficient is around ~ 0.5 $\mu m^2 s^{-1}$ [166]. The N-type calcium channels are the multimeric complex (up to 492kDa) of the main pore forming and auxiliary subunits[10]. As far as it is known that there is the correlation between the size of proteins and diffusion coefficient[161] I assumed that the diffusion coefficient for N-type calcium channels cannot be greater than 0.1 $\mu m^2 s^{-1}$. To relationship between the diffusion coefficient (D) over distance can be described by the following equation;

$$D = x^2/(2t) \quad [2.4]$$

where: x^2 is mean square displacement of particles at given time (t)[162]. Based on this relation the mean speed of N-type calcium channel has been accepted as ~ 0.45 $\mu m/sec$. There are dozens of available software tools for particle tracking[167], although at the beginning I have decided to use Automated single particle detection and tracking software for large microscopy datasets written by one of our lab colleagues[168].

A second software chosen for single N-type calcium channel tracking was written in Matlab (The Mathworks, Inc.; Natick, MA) by Isabel Schlangen[170] (Heriot-Watt University). The detection of the particles is based on the à trous wavelet transform[171] which separates the molecules from the background based on their size. The next step uses the Probability Hypothesis Density (PHD) filter[170,172]. This filter is a two-stage iterative process that predicts the next location of all objects based on a suitable motion model and then updates this belief based on the incoming detections in the current image frame (figure 2.5). As an output of the filtering, we get the list of detected and linked molecule positions which can be displayed on the raw image stack in Fiji using the MTrackJ plugin[173].

The algorithm was tested on simulated data set of the ISBI 2012 Particle Tracking Challenge[167]. Scenario 3 with receptors was chosen as the similar dynamics can be expected to the voltage-gated calcium channels. The trajectories of objects are created with two switching motion behaviours; Brownian motion and constant velocity. The simulated images of 100 frames and size 512x512 pixels with SNR of 3 and low density were chosen. Representative frames are shown in figure 2.6.
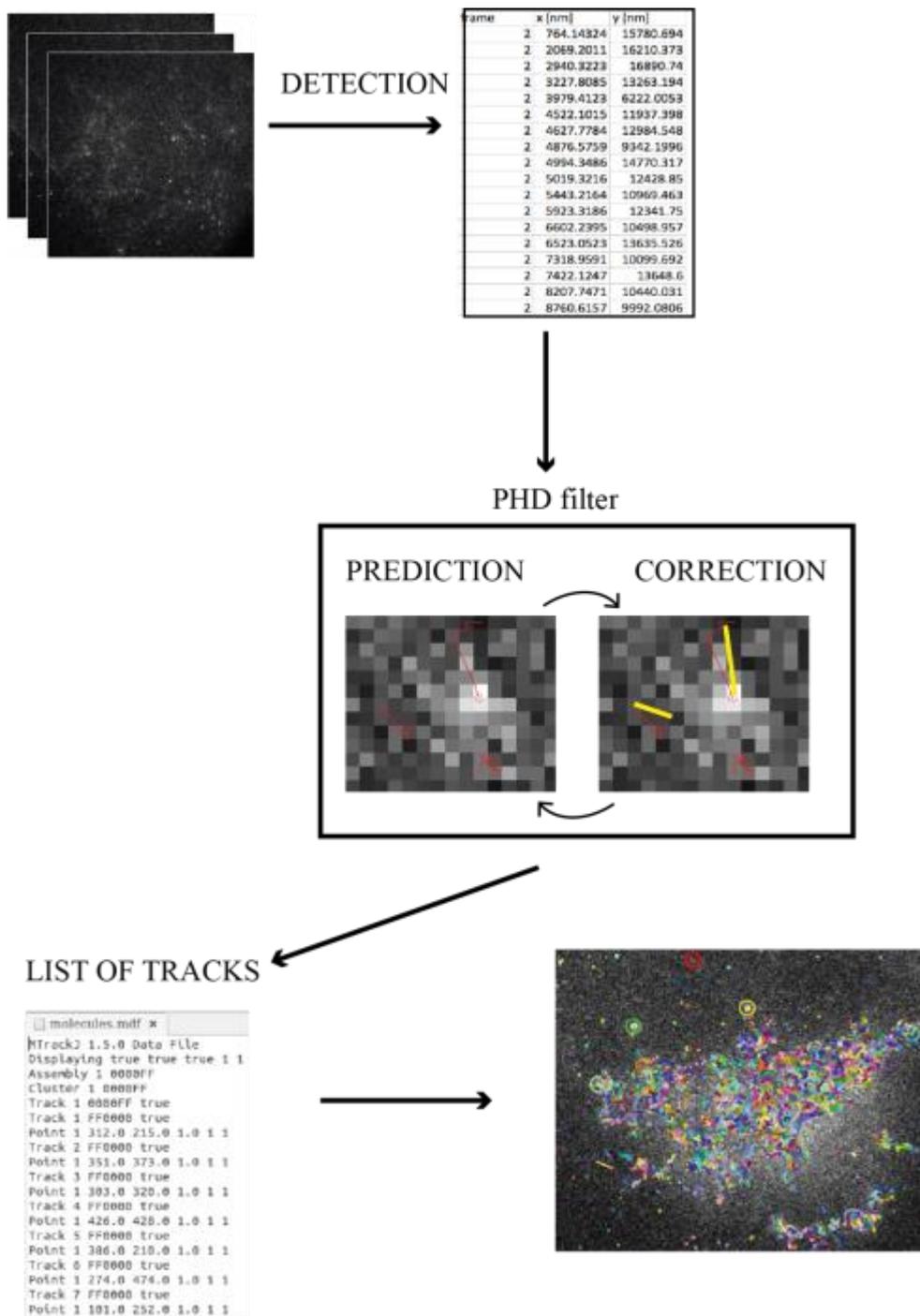
Figure 2.5 A flowchart illustrating the main steps of single-particle tracking. Molecules are separated from the background based on their size. The next step uses the Probability Hypothesis Density (PHD) filter[170,172]that predicts the next location of all objects and then updates this belief based on the incoming detections in the current image frame. As an output of the filtering we get the list of detected and linked molecules.
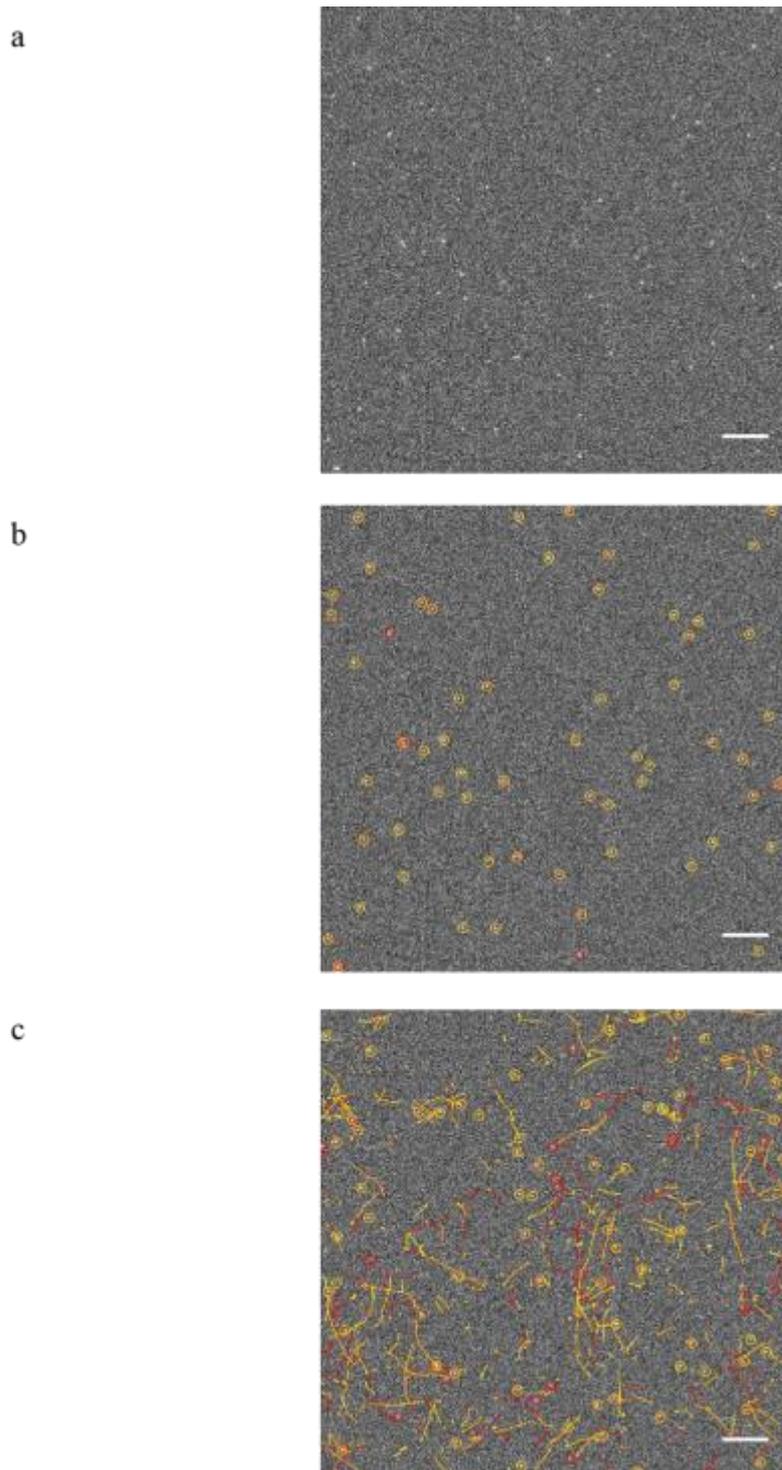
a

b

c

Figure 2.6 Simulated data sets of the ISBI 2012 Particle Tracking Challenge tested with Matlab Particle Tracking algorithm. Scenario 3 with receptors SNR of 3 and low density was chosen. (a) raw data, (b) detected molecules, (c) trajectories of linked molecules. Red represents ground truth and yellow represent extracted detections (scale bar 50pix).

2.7.6 R-Geco imaging

The activity of N-type calcium channels was recorded by combining the patch clamp technique with TIRF microscopy (Figure 2.7 a). This method is called *optical patching*[174]. The experiments were done in collaboration with Dr Euan Brown and Matthew Euston (Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt University, Edinburgh). The acquisition was performed by imaging the R-Geco-SNAP25 with 561nm laser excitation in TIRF mode, using Olympus IX-81 TIRF+ microscope with 1.4 NA 150x immersion oil lens. An EMCCD camera (Hamamatsu. UK) was used, which has a pixel array of 512 x 512, resulting in a pixel size of 106nm. The source of excitation was a 100 mW laser diode. The exposure time was 60ms. The TIRF angle was adjusted using the Olympus software Xcellence, setting the correction angle for each laser excitation beam individually. The micro cell incubator box surrounding the microscope had to be removed, which meant that cells were imaged at room temperature, (22°C), with a nosepiece stage to hold the sample. The biological samples were prepared using either PC12 cells or Att20 cells transfected with R-Geco- SNAP25. R-Geco is a genetically encoded calcium indicator based on calmodulin, M13 and two fluorescent proteins[175,176]. This indicator can be targeted to membrane proteins, which enables to image calcium activity at the membrane (Figure 2.7 b). The high affinity of calmodulin to calcium channels results in an increase of fluorescence absorption and emission. Both the imaging and the voltage clamping were synchronised in time. Five different steps for 30sec were registered. The first step was a control with -80mV, which is the membrane resting potential. Every calcium channel at the membrane was closed. During the second step, a current of -30mV was measured. In this stage only L-type calcium channels were open. The last two steps have shown depolarisation of the membrane with a current of 0mV and +20mV. L-type and N-type calcium channels were open. The last step shows the current from -80mV to 20mV.
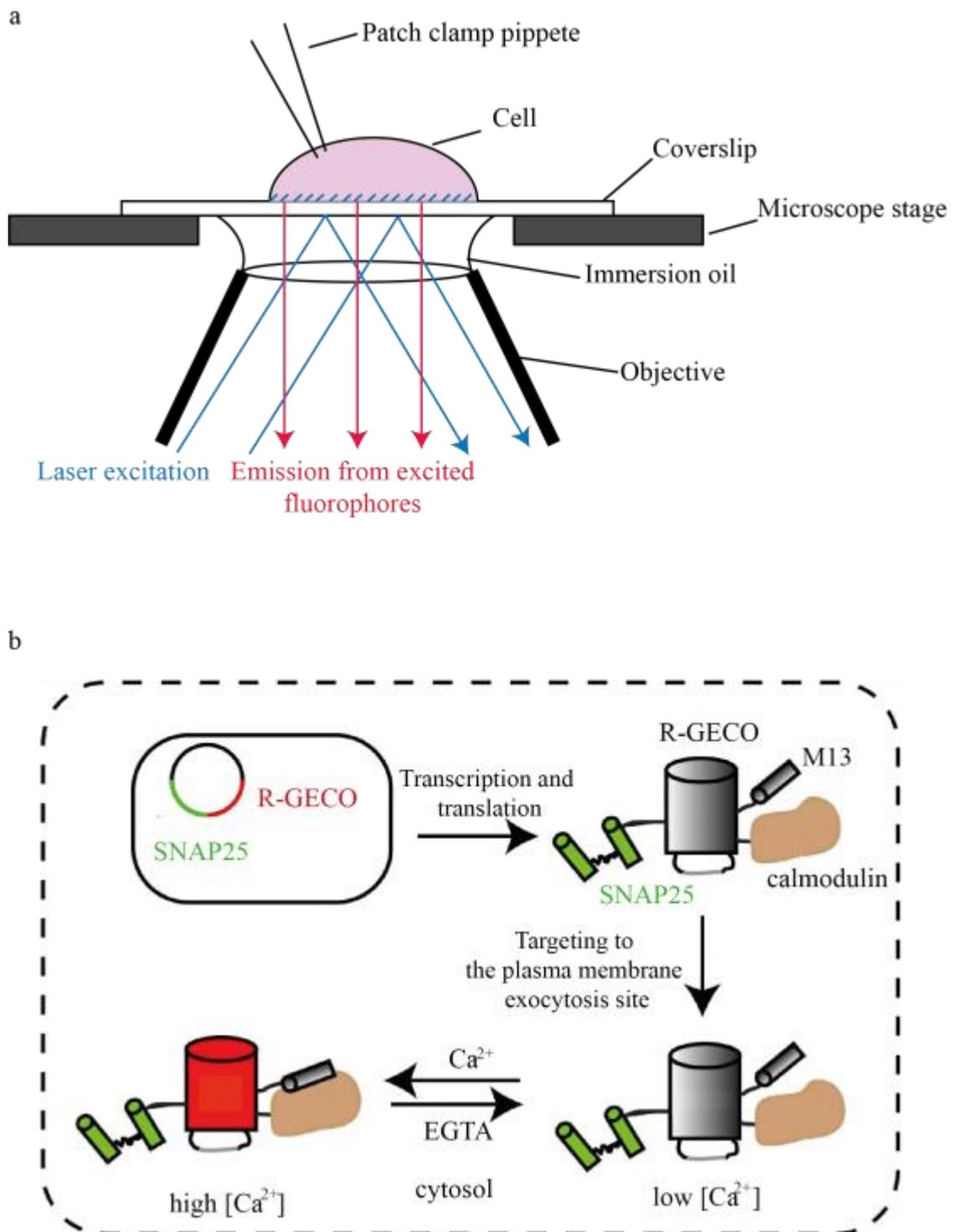
Figure 2.7 R-Geco imaging. (a) Schematic diagram of optical patching setup. (b) Cartoon representation calcium dependence. The high affinity of calmodulin to calcium channels results in an increase of fluorescence absorption and emission.

2.8 Statistical analysis

Results presented in this thesis were analysed with non-parametric statistical tests for unpaired groups. To compare two groups the Mann-Whitney U test was used and for three or more, the Kruskal-Wallis test was performed. The statistical significance is demonstrated by * for p values between 0.005-0.05, ** for p values between 0.0005-0.005, *** for p values between 0.0001-0.0005 and **** for $p < 0.0001$.