CHAPTER FIVE:

ENDOGENOUS VOLTAGE-GATED CALCIUM CHANNEL ACTIVITY

5.1 Introduction

As described in chapter one (1.2.2) voltage-gated calcium channels play a crucial role during exocytosis. Vesicles that have accumulated at the plasma membrane are triggered to fuse by a rise in local free Ca^{2+} ions. During the resting membrane potential, the intracellular concertation of Ca^{2+} is lower (50-100 nM) than the extracellular (1.8 mM). Changes in the membrane potential increase the likelihood of opening of voltage-gated calcium channels and thus generate a rise of Ca^{2+} ion concentration up to 200 μM[77,78] close to the channel mouth. The areas in cell cytoplasm whit localised high Ca^{2+} concentrations are called microdomains[177]. The distribution of voltage-gated calcium channels and the size of microdomains have an influence on the exocytosis process. The process of calcium ion entry and releasing the vesicle is very fast, estimated to be in the range of 200 μs[76]. It is very important to understand not only the distribution of voltage-gated calcium channels but also their activity. Electrophysiology techniques, like patch clamp, allow measurement of ion channel activity. Using this set up, we can record either single calcium channel or ensembles of channels over time.

It is also possible to observe the activity and distribution of the calcium channels of the entire cell. The combination of these two biophysical techniques allows the measurement of the activity ion channels in single cells with high spatial and temporal resolution. In this chapter, I describe the experiments that combine electrophysiology techniques with TIRF microscopy. The activity of voltage-gated calcium channels was examined using different pharmacological interventions. By using the genetically encoded calcium indicator (R-Geco-SNAP-25) and TIRF microscopy simultaneously the aim of this chapter was to create an activity map of voltage-gated calcium channels at the cell membrane. It is hypothesised that different subtypes of calcium channels can be distinguished by comparing their activity (the intensity of fluorescence over time).

Experiments combining patch clamp with TIRF microscopy were performed in collaboration with Dr Euan Brown and Matthew Euston (Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt University, Edinburgh).

5.2 Expression of R-GECO-SNAP-25 at the cell membrane

Although the activity of voltage-gated calcium channels can be recorded by using organic dye calcium indicators[174,182], there are a lot of disadvantages due to the cellular localization of indicators. The problem may be addressed by using genetically encoded calcium indicators that can be targeted directly to the compartment of interest. Here, in my thesis, I have used the genetically encoded calcium indicator R-GECO and SNAP-25 targeted to the plasma membrane (chapter two 2.6.6). R-GECO is a red fluorescent sensor, developed in Robert Cambell's lab[176], where the fluorescence intensity increases in response to increased intracellular calcium concentration[176]. It is most sensitive to concentration changes between 100 nm to 1 µM[176], which makes it a potentially suitable calcium indicator for experiments related to exocytosis.

To verify the plasma membrane targeting, the AtT-20/D16-16 cells were transfected with R-GECO-SNAP-25. The imaging was performed as described in chapter two (2.6.6) in 2mM Ca^{2+} warm (37°C) Ringer Buffer. To test the response of the R-GECO-SNAP-25 to intracellular calcium concentration, cells were treated with 1 µM ionomycin. Ionomycin is an antibiotic that acts as an ionophore[183,184], that binds Ca^{2+} ions (in a 1:1 stoichiometry) and transports them across the cell membrane avoiding the endogenous entry pathways. Here it was used to increase the intracellular concentration of Ca^{2+} ions to that equilibrating with the external solution.

The widefield images (figure 5.1a) indicate the R-GECO-SNAP-25 plasma membrane localisation. There is an increase in fluorescence intensity after adding 1 µM ionomycin (figure 5.1b). Then the cells were imaged using TIRF without adding ionomycin (figure 5.1c). Although the fluorescent intensity is very low, the single on and off events can be recorded (figure 5.1d). After the fluorescence intensity for each single event was measured, there is a short increase in fluorescence for few frames. These results show that the R-GECO-SNAP-25 construct is targeted to the cell plasma membrane, and the fluorescence intensity increases with increasing Ca^{2+} ion concentration.

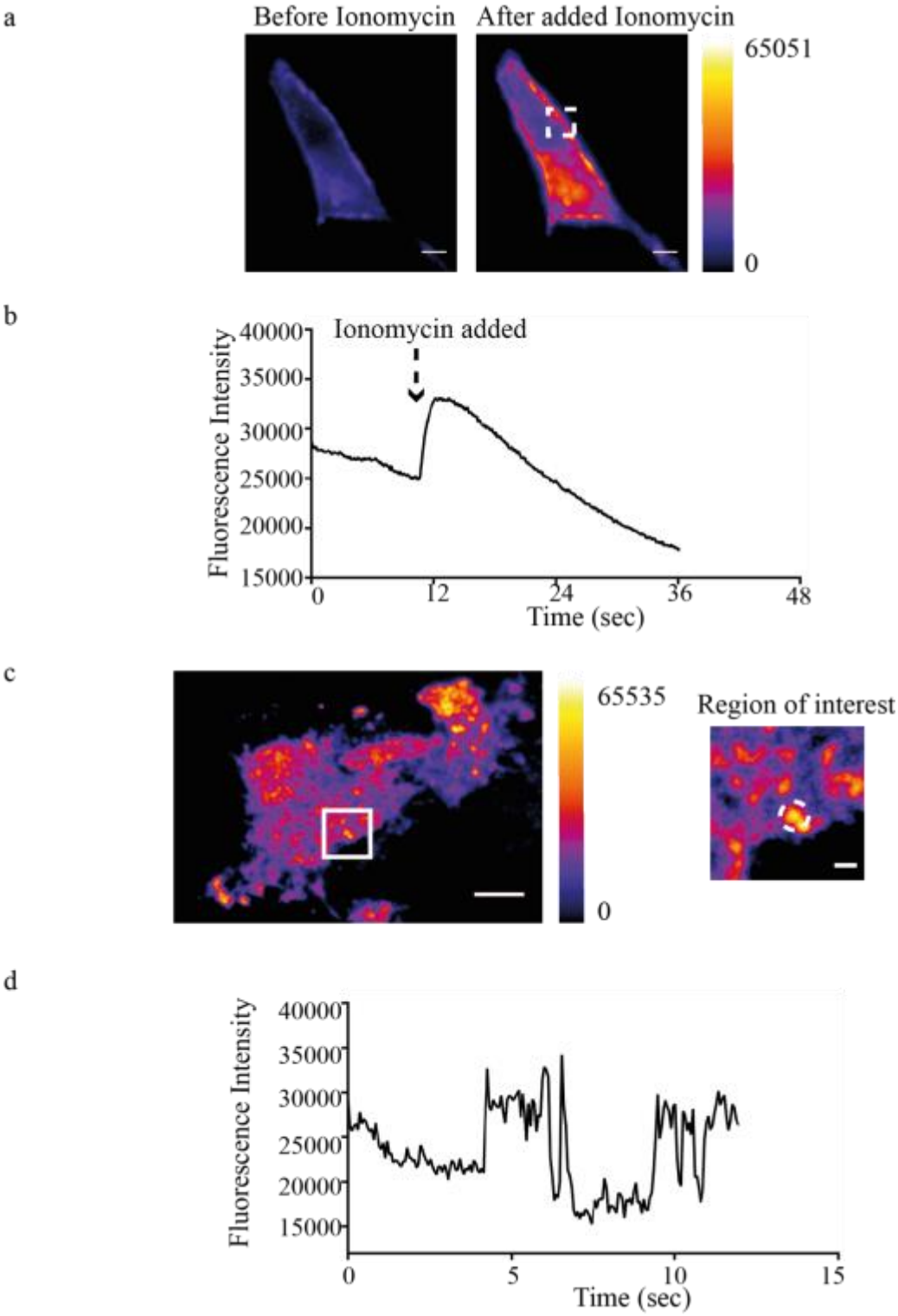

Figure 5.1 Expression of the R-GECO-SNAP-25 at the cell membrane. AtT-20/D16-16 cells transfected with R-GECO-SNAP-25 were treated with 1 µM ionomycin in a Ringer Buffer. (a) Widefield images of the representative cell before and after added ionomycin. Scale bar 5 µm. (b) The fluorescence intensity profile at the plasma membrane of the ROI indicated in (a). Scale bar 5 µm (ROI 1 µm). (c) TIRF images of the representative cell after added ionomycin. (d) The fluorescence intensity profile at the plasma membrane of the ROI indicated in (c).

5.3 Analysis of single Ca^{2+} microdomains

Single R-GECO on and off events at the cell membrane are hypothesised to be triggered by the opening of single or cluster calcium channels and local intracellular changes in Ca^{2+} ion concentration during calcium channels opening. The dimension of microdomains depends on the amount of Ca^{2+} entry but also on the single-channel current amplitude[185]. There is a proposed model,[79] describing the relationship between the depolarization and single calcium current. The concept says that small depolarizations of the membrane can open only a few voltage-gated calcium channels, but the ensemble calcium current is large. When the depolarization is large, it activates many voltage-gated calcium channels, but the single calcium current is small. The single-channel currents vary between the channel types. The cell type that has been used for experiments in this chapter contains different voltage-gated calcium channel types. Whole-cell voltage-clamp of AtT-20 cells (measuring cell capacitance and current density) determined 65 % of L-type, 11.5 % of N-type and 23.5 % of other calcium channel types (measurements carried by E. Brown and M. Euston). The total number of voltage-gated calcium channels at the cell membrane was estimated to be 13,000 in each cell, that gives the 8.6 voltage-gated calcium channels per μm^2 (cell surface area 1500 μm^2). Assuming that only ~40% of the cell membrane can be seen using TIRF microscope, only around 5,200 channels can be recorded. The voltage-gated calcium channels differ between type not only by single-channel currents but also by inactivation time. L-type calcium channels in embryonic chick heart cells can be open for >20 ms[186]. The N-type calcium channels have faster inactivation time, they stay open for a shorter time. These properties provided me with a potential way to distinguish different channel subtypes in single cells based on the image properties their signals generate.

Cells were analysed using the F÷F0 Fiji plugin[187]. This plugin corrects the irregular fluorescence intensity (F) by normalizing the fluorescence against resting fluorescence (F0) and highlights fluorescence intensity changes as a ratio between the rolling image train and the initial intensity values. Each calcium event (calcium puffs, spots) was picked manually and analysed with the fluorescence intensity profile. By measuring the FWHM of the activity duration, the time of the Ca^{2+} event was calculated (figure 5.2) according to the frame rate (60 ms). The number of single events picked for each cell in control samples was around 40. The FWHM of activity duration varies between spots. They can be divided into two groups: short activity duration (below 200 ms) and long activity duration (over

200 ms) (figure 5.2c). The experiments described below, are aimed to be able to distinguish different type of calcium channels based on the length of their opening time.

5.4 Calcium activity after calcium channel agonist and blockers

To test the activity duration for different calcium channels type in AtT-20 cells, different channel blockers or agonists were used. To check the spatial calcium channel activity, cobalt was added to the imaging buffer. Cobalt ions added to external buffer block voltage-gated calcium channels[188]. Thus, $CoCl_2$ solution was added to the cells in Ringer Buffer to obtain the final concentration 2 mM Co^{2+}. Cells were imaged 10 min after adding of $CoCl_2$ (figure 5.3). Images of treated cells with cobalt were analysed and compared to control cells. The number of single events were always less than 10 per cell after this treatment.

Next, I tested a known L-type calcium channel agonist. BayK K8644 increases the single channel current which results in increased channel open time[189,190]. The BayK was added to the cells in the Ringer Buffer to obtain final concentration 1 µM. Cells were imaged 5 min after BayK treatment and images analysed and compared to control cells as before. There was no difference in a number of events in cells treated with BayK, but it was clearly seen that the length of activity increased compared to the control cells (figure 5.4).

The known L-type calcium channel blocker Nifedipine blocks 90% of L-type calcium channels[190,191]. Thus, Nifedipine was added to the cells in the Ringer Buffer to obtain final concentration 1 µM. Images were analysed as before, and compared to control cells (figure 5.5). The number of single events decreased significantly.

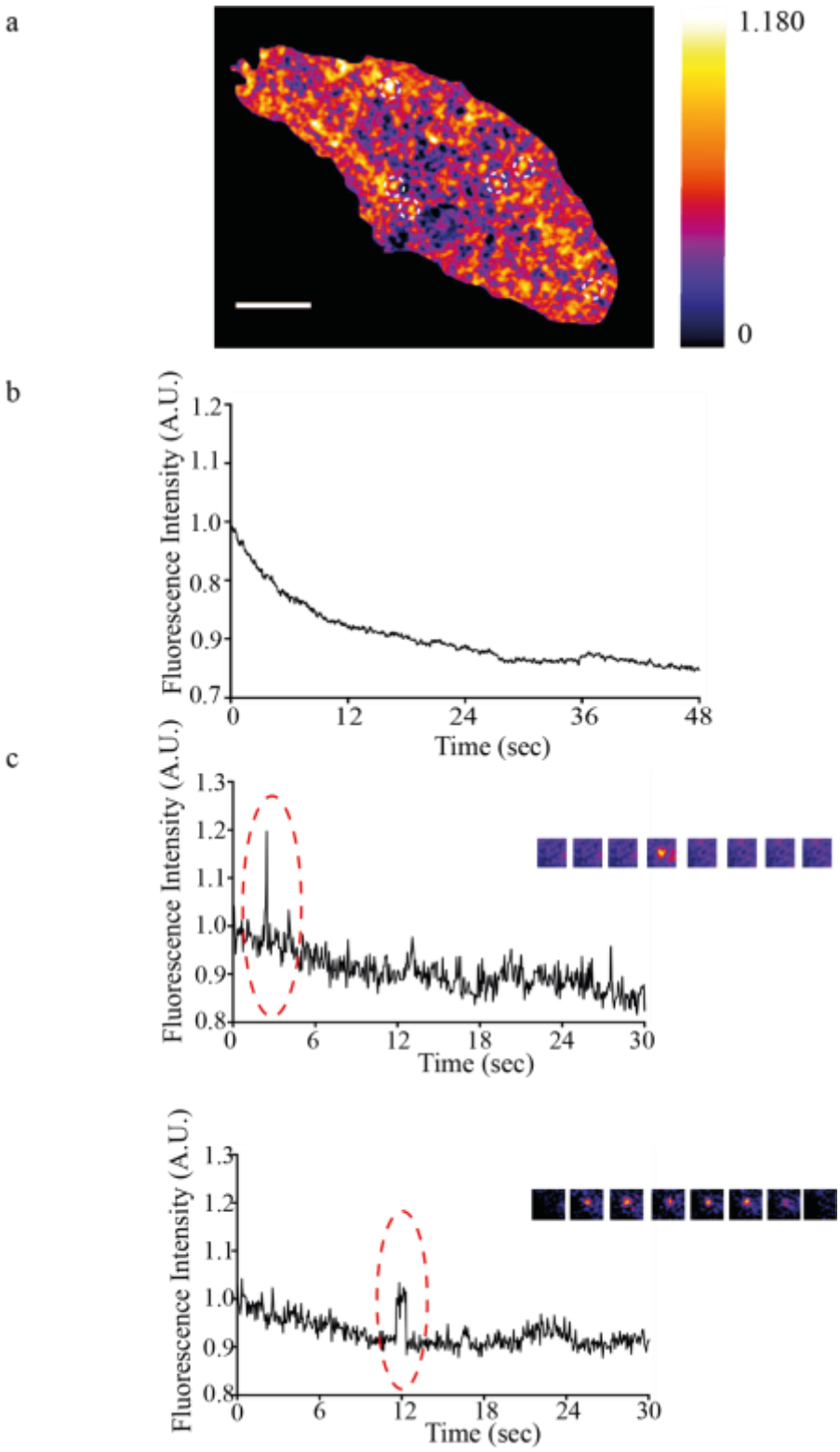

Figure 5.2 Spatial calcium activity in control cell. (a) TIRF image of a representative cell. Scale bar 5 µm. (b) The fluorescence intensity profile of the whole cell. (c) The fluorescence intensity profile of a (2 x 2 µm) ROI indicated in (a) represents different types of activity duration.

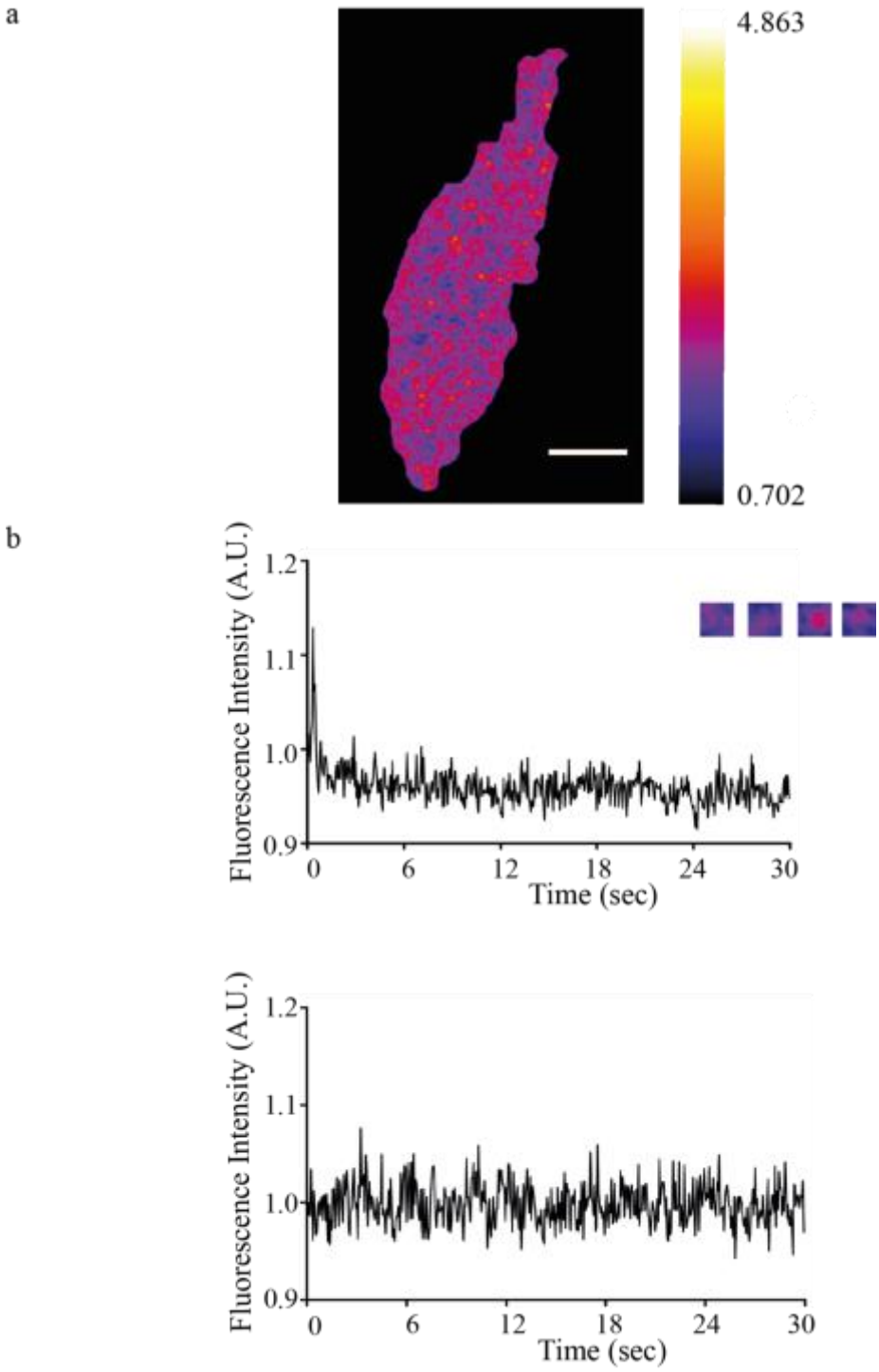

Figure 5.3 Calcium activity after added cobalt. (a) TIRF image of a representative cell. Scale bar 5 µm (b) The fluorescence intensity profiles of (2 x 2 µm) ROIs represent no activity.

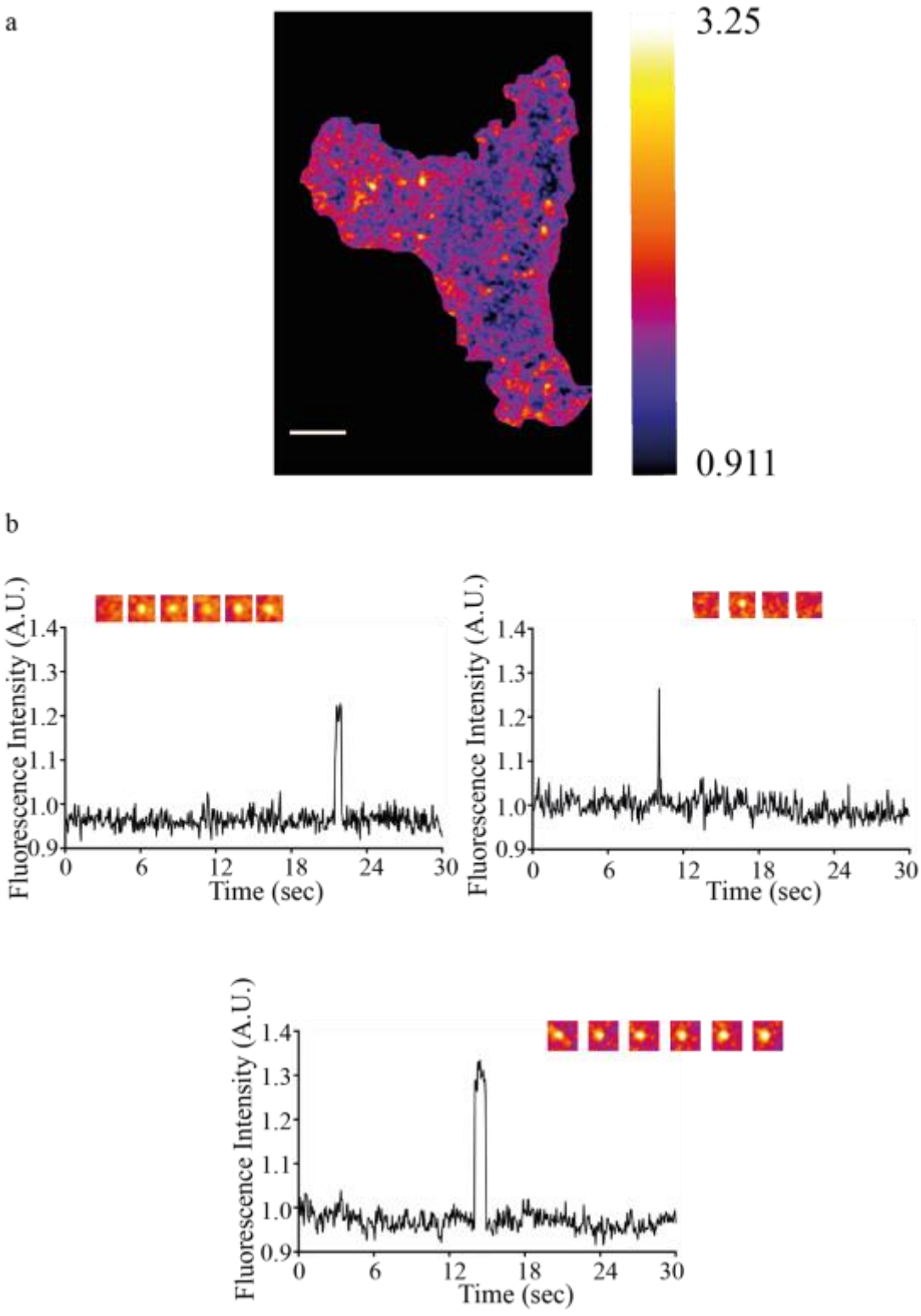

Figure 5.4 Calcium activity after added BayK. (a) TIRF image of a representative cell. Scale bar 5 µm. (b) The fluorescence intensity profiles of (2 x 2 µm) ROIs represent different calcium activity.

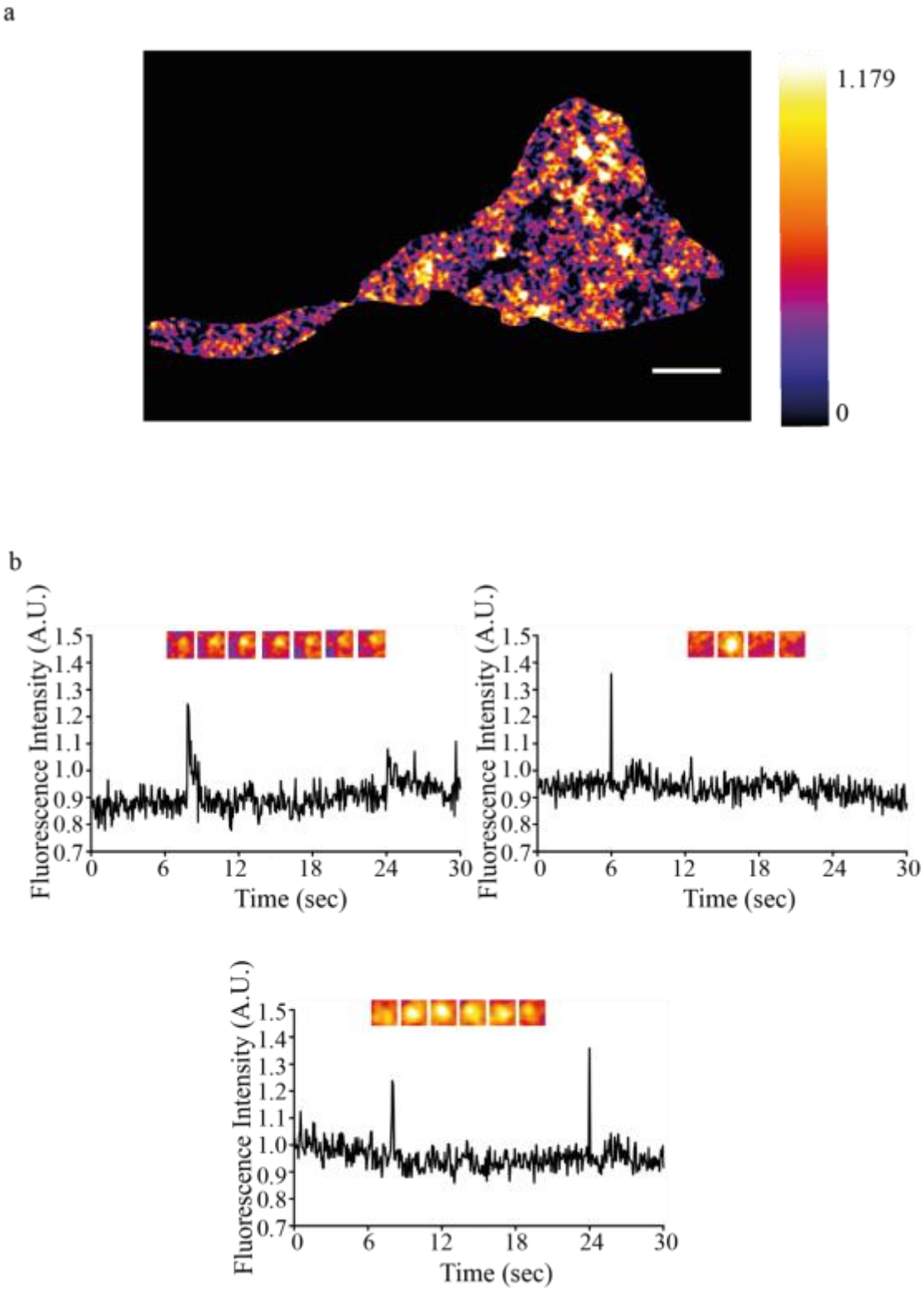

Figure 5.5 Calcium activity after added Nifedipine. (a) TIRF image of a representative cell. Scale bar 5 µm (b) The fluorescence intensity profiles of (2 x 2 µm) ROIs represent different calcium activity.

To compare the difference in events length for different drugs, the non-parametric Kruskal-Wallis test for multiple comparisons was used. The box-and-whisker plot of the FWHM of activity duration (in seconds) for control and after treatment with 1 µM BayK and 1 µM Nifedipine is presented in figure 5.6a. The median FWHM activity time of the calcium event in control sample is 0.1200 second. In comparison, the median time after added BayK is higher (0.2280 sec) and after added Nifedipine lower (0.1005 sec) (figure). After statistical comparison of groups, there is a significant difference between control and BayK and between BayK and Nifedipine. There is no significant difference between control and Nifedipine (figure 5.6a).

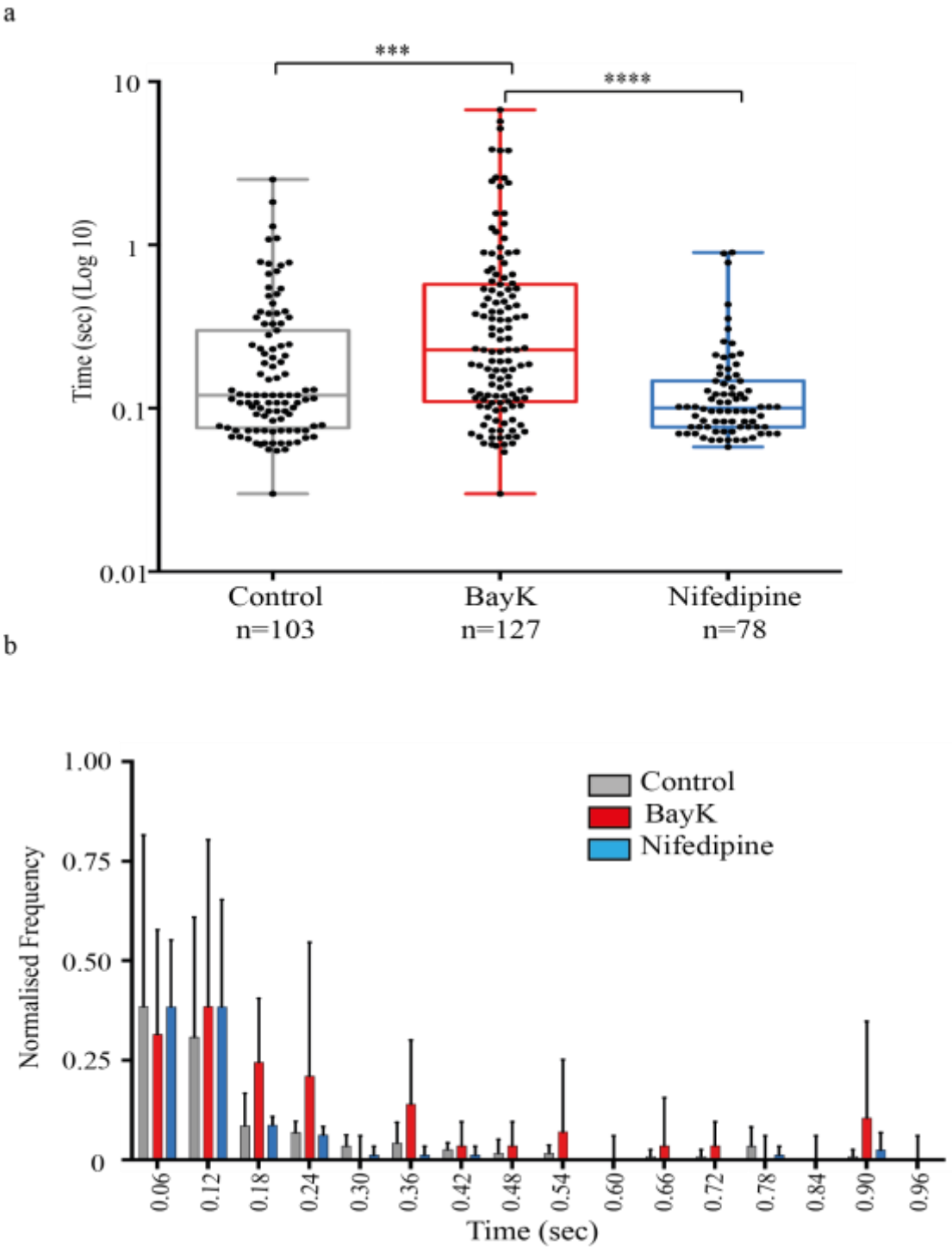

Figure 5.6 Comparison of calcium activity after treatment with drugs. (a) Boxplots of the FWHM activity duration in a second (log10 scale). (n= number of events). Statistical significance was verified using the non-parametric Kruskal-Wallis test for multiple comparisons. The centre line indicates median value, the boxes 25th and 75th quartile values, and whiskers minimum and maximum values. (*** is p = 0.0002, **** is p < 0.0001). (b) Normalised frequency histogram of mean ±SD. The number of cells for each group =4.

5.5 Optical patching

The patch-clamp method allows demonstrating the existence of different calcium channel types at the cell membrane, due to different kinds of single channel activity[192]. To examine calcium channel behaviour, different membrane potentials were recorded alongside TIRF imaging of R-Geco-SNAP-25 in AtT20 cells. This experiment aimed to increase understanding of calcium channel behaviour in response to voltage changes. The control sample was recorded with a holding potential of -80mV, when voltage-gated calcium channels should be predominantly closed. Next, the cell membrane was depolarized at -30 mV which activates the L-type calcium channels[192]. For activation of both N-type and L-type calcium channels, 0mV and +20mV depolarization was used[192] (figure 5.7). To analyse the calcium events, images have to be acquired with high spatial resolution. The box-and-whisker plot of the FWHM of activity duration (in seconds) for three different membrane potentials is presented (figure 5.8a). The median value for all membrane potentials is 0.1 sec. The statistical difference was tested using the non-parametric Kruskal-Wallis test for multiple comparisons. The results show no significant difference between different membrane potentials, although those result would have to be compared to more cells.

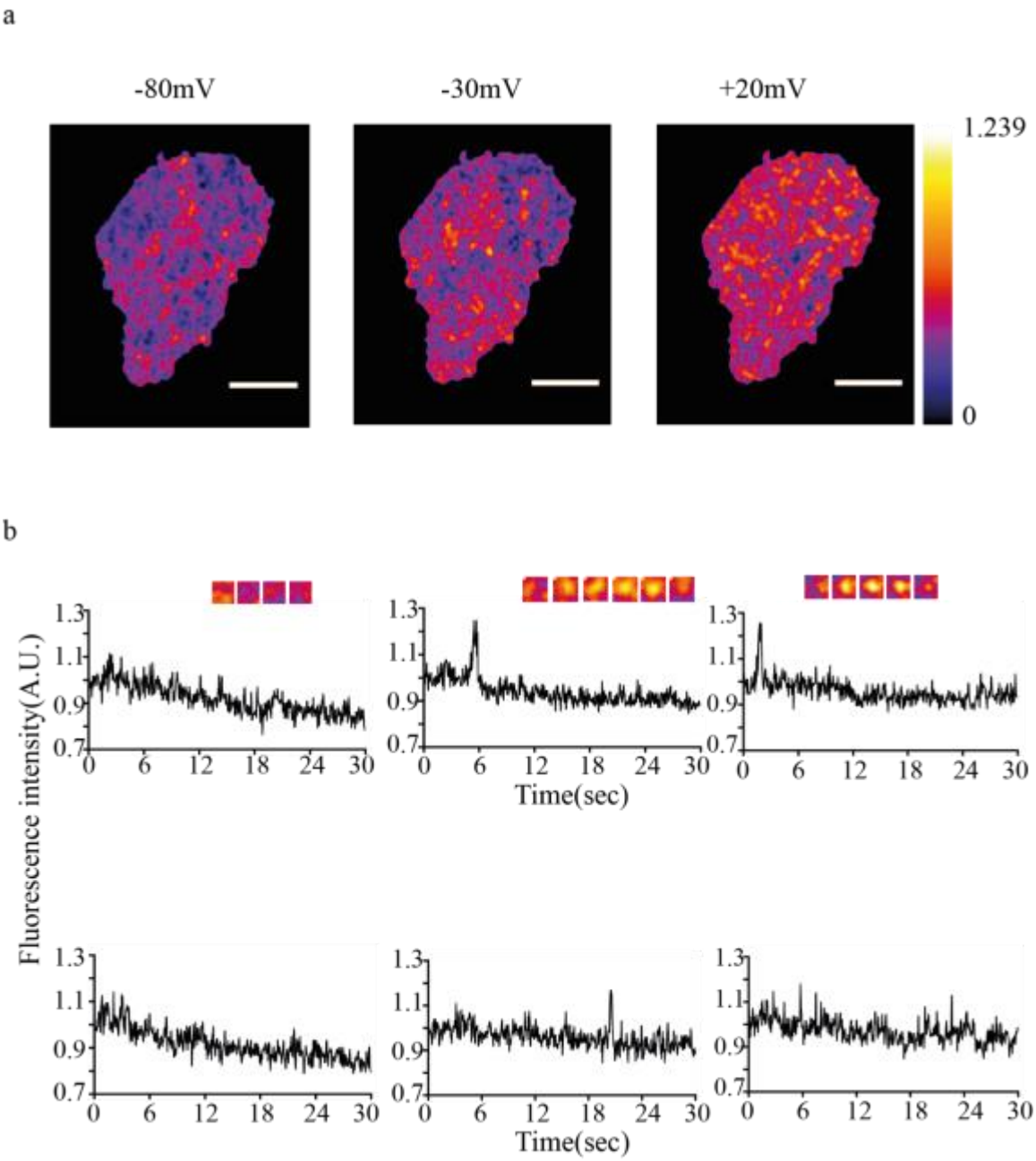

Figure 5.7 Calcium activity at different membrane potentials. (a) TIRF image of the cell with different membrane potentials. Scale 5 µm (b) The fluorescence intensity profile of representative calcium events for holding potential (-80 mV), and depolarisation (-30 mV and +20 mV).

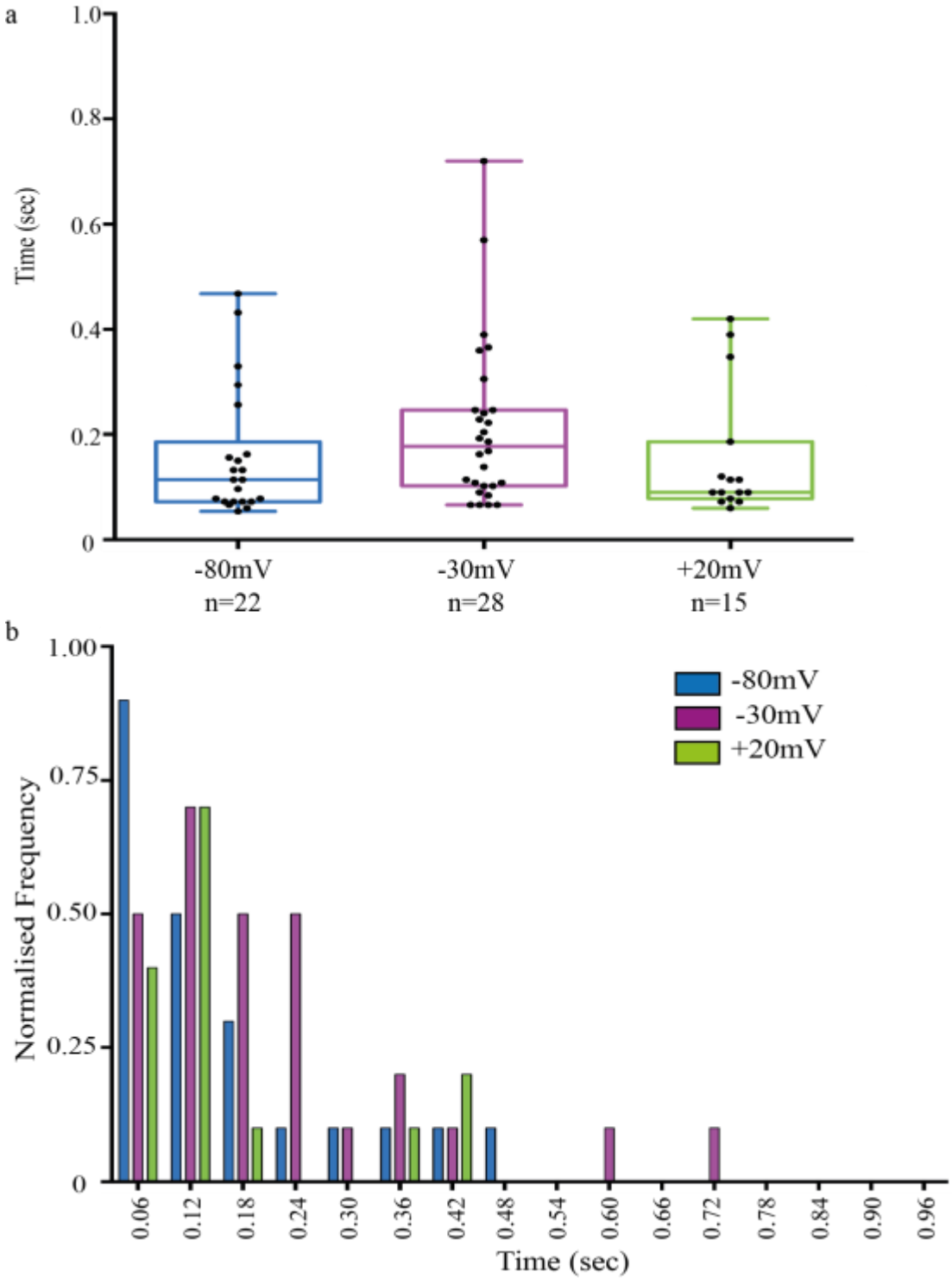

Figure 5.8 Comparison of calcium activity for different membrane potentials. (a) Boxplots of the FWHM activity duration in a second. (n= number of calcium events). Statistical significance was verified using the non-parametric Kruskal-Wallis test for multiple comparisons. The centre line indicates median value, the boxes 25th and 75th quartile values, and whiskers minimum and maximum values. (b) Normalised frequency histogram of mean. The cell number =1.

5.6 Conclusions

Calcium indicators are an invaluable tool for imaging intracellular Ca^{2+} signals with high spatial and temporal resolution. The genetically encoded calcium indicator R-GECO-SNAP-25, allows for high-resolution analysis of calcium channel activity at the cell membrane. The experiments with drugs aimed to distinguish different type of voltage-gated calcium channels and analyse their spatial activity. The differences in medians after treating with drugs, support the hypothesis that the different types of calcium channels can be characterized by the time of their opening. After treating with L-type calcium channels agonist, BayK, the number of longer activity durations increased, while after treatment with L-type channels blocker, this parameter decreased. The combination of electrophysiology with high-resolution microscopy would be a powerful technique providing the spatial information of channels distribution and activity.

The number of block calcium events increased after treatment with BayK and decreased after adding Nifedipine. This result could validate the real number of calcium channels at the AtT20 cell membrane. The difference in the number of calcium events was analysed with non-parametric Kruskal-Wallis statistical test. There is no significant difference in the number of calcium events between the control and BayK, or the control and Nifedipine, but there is a significant difference between BayK and Nifedipine. The majority are L-type calcium channels, which are more likely to open after treated with BayK, while after adding L-type channel blocker-Nifedipine, there is lower likelihood to see calcium events. The number of events after changing the membrane potential cannot be compared because only one cell was analysed.

By analysing the behaviour of calcium channel openings, we have to keep in mind the slow kinetics of R-GECO calcium indicator and imaging speed limited by camera frame rate. The process of calcium channel opening and closing results in changes of Ca^{2+} concentration ~200 μM in very short time (<1ms)[193]. By measuring the kinetics of calcium channels opening we have to keep in mind that the genetically encoded calcium indicators have slower kinetics than chemical calcium indicators. The binding rate constant (K_a) is defined by Ca^{2+} binding rate over Ca^{2+} dissociation rate, which for R-GECO is ~12 ms[194,195]. For the R-GECO-SNAP-25 imaging, an EMCCD camera was used. Although this type of camera is slower (31 ms per frame) than the sCMOS camera (10 ms per frame), the EMCCD has a higher signal-to-noise ratio than sCMOS[101], which is big disadvantage when

the sample is photon-restricted.

The calcium event analyses were performed manually. This method is insensitive to small intensity changes, that can be not noticed by eyes. The calcium activity has to be still analysed with an automated script developed and written by Dr Paul Dalgarno (Institute of Biological Chemistry, Biophysics and Bioengineering, Heriot-Watt University, Edinburgh). This method is based on autocorrelation analysis. Autocorrelation compares the similarity between detected fluorescent intensity profiles as a function of the time lag between them[201]. It can detect small and gradual changes in calcium events. That kind of analysis has been tested before on idealised synthetic data[202], but has not been applied to real data such as those presented here.

The methods developed and presented in this chapter when optimised could be a screening system for calcium channel-related drugs and to monitoring the changes of calcium ions in cells.

The drawback of the techniques developed here is that only single or a few cells at one time can be observed, making these approaches inherently low-throughput and so unsuitable for screening applications. One novel tool that may have the potential to improve biological throughput is turning living cells into a laser. The first time this technique was demonstrated by Gather et al in 2011[196]. Because of the improved higher signal to noise ratio compared to stimulated emission, lasers have the potential for faster techniques that can be used by industry to rapidly test drugs. As an improvement of calcium imaging, I worked in collaboration with Mark McKenzie and Prof. Ajoy Kar from the Institute of Photonics and Quantum Science, Heriot-Watt University. This project was focused on creating novel tools for the generation of light from something that is living.

Laser stands for Light Amplification by the Stimulated Emission of Radiation. A laser beam is a special form of light. Most lasers are built using a cavity filled with excitable atoms and with a mirror at each end. The atoms can be in a gas form such as CO_2, solids like rubies, or even liquids containing dyes. The laser is ‘pumped’ by adding energy to the cavity, in the form of the flash of light, chemical reaction or electrical energy, a spark or constant current. Photons are spontaneously emitted from the excited atoms in all directions with most of these absorbed by the walls of the cavity except those bouncing back and forward between two mirrors at each end. Bouncing atoms hit other atoms on their way and

they amplify themselves. One of the mirrors is designed to reflect only 95-99% of the light hitting it, allowing the other 1-5% to exit the cavity as a laser beam. Because the photons travel in the same directions the beam of the laser is parallel and doesn't spread out like the beam of the torch. The laser beam is so straight that the light and its energy can be focused onto a very small point, for example, a single cell[197].

In our experiment, HEK293 cells suspended in the Ringer Buffer, loaded with the fluorescent dye Calcein Acetoxymethyl (Calcein AM) were used as a gain medium. Cells were placed in the laser cavity formed from two plane mirrors with peak reflectivity at 532 nm. When cells were excited with 474 nm laser pulsed at 100 fs repetition rate, they started to emit light (figure 5.9a). After adding ascorbic acid which is an antioxidant, the lifetime (i.e. the time before emission stopped, presumably because of photodamaged) was increased from 60,000 to 130,000 pulses. Ascorbic acid has been used to decrease photobleaching of fluorescent dyes by quenching radical species[198]. The next step of this experiment was to apply this method to a biological assay. To observe calcium signalling in the cells, the fluorescent calcium indicators such as Fluo-3 or GCamp-SNAP-25 were used (figure 5.9b,c). Fluo-3 is an intracellular calcium (Ca^{2+}) indicator which is non-fluorescent, but after binding to calcium ions it becomes fluorescent. Fluo-3 is an AM ester which permeates cell membranes as uncharged molecules. Inside the cell, esterase cleaves the AM groups resulting in the binding of Fluo-3 to calcium ions. In this case, the dye is everywhere in the cell. The second calcium indicator GCamp-SNAP-25 is a genetically encoded calcium indicator fused to the SNARE protein SNAP-25, which only localises at the plasma membrane. To detect the fluorescent intensity changes due to changes in calcium ion concentration the CagedCa^{2+} was added to the cells in Ringer Buffer. CagedCa^{2+} (NP-EGTA (o-Nitrophenyl EGTA, Tetrapotassium Salt)) is a photolabile AM ester chelator, which binds free calcium ions from the experimental buffer and permeates the cell membrane. Once inside the cell, intracellular enzymes, esterase, converts it to NP-EGTA which becomes a photolabile chelator with a high selectivity for Ca^{2+} ions. Triggered by a UV flash (405 nm) its K_d increases from 80 nM to >1 mM[219] and the caged ions are uncaged and released inside the cell, which results in an increase of Ca^{2+} ions concentration within the cell[199]. At the beginning, the experimental set up was tested using a widefield microscope in TIRF mode. AtT-20 cells were loaded with Fluo-3 and CagedCa^{2+} and imaged using a 491 nm laser. The small region in the cell was irradiated with the 405 nm laser for multiple cycles to release free Ca^{2+} ions from CagedCa^{2+} (figure

5.9b). HEK293 cells transfected with GCamp-SNAP-25 and loaded with CagedCa^{2+} were analysed with the same settings (figure 5.9c).

The fluorescence intensity increased after exposure to 405 nm laser (figure 5.9c,d). The experiment was repeated with cells in suspension on a microscope slide using an Argon 488 nm laser. After irradiating with a UV laser (405 nm) the fluorescence intensity increased (figure). For the last step of the experiment, the cells have been placed in Ringer Buffer into a cavity, between two mirrors and the emission of the calcium indicators was recorded. Unfortunately, after many trials, it was not possible to register any emission from the cells. The concentration of loaded or transfected indicators was increased, the cells attached to the bottom mirror were tested, but nothing gave positive results. The signal from Fluo-3 is very weak, and the experimental set up has to be improved. Cells were monitored using a viability indicator Propidium Iodide (PI).

The two-photon laser method used for the experiment is less cytotoxic for the cells than methods described before[196]. Adding an antioxidant to the sample increased their lifetime and emission lasts up to 2 minutes before the dye was photobleached. Laser emission is monochromatic, which results in an improvement of the signal to noise ratio. Under the microscope, the fluorescent dyes emit light randomly in all direction, here using cells in the cavity we have obtained a coherent beam light. The emission peak of the laser was much higher than the fluorescence, which improves the detection of low signals. After improving the laser set-up this approach could be a valuable technique to examine cells and could be used in the pharmaceutical industry.

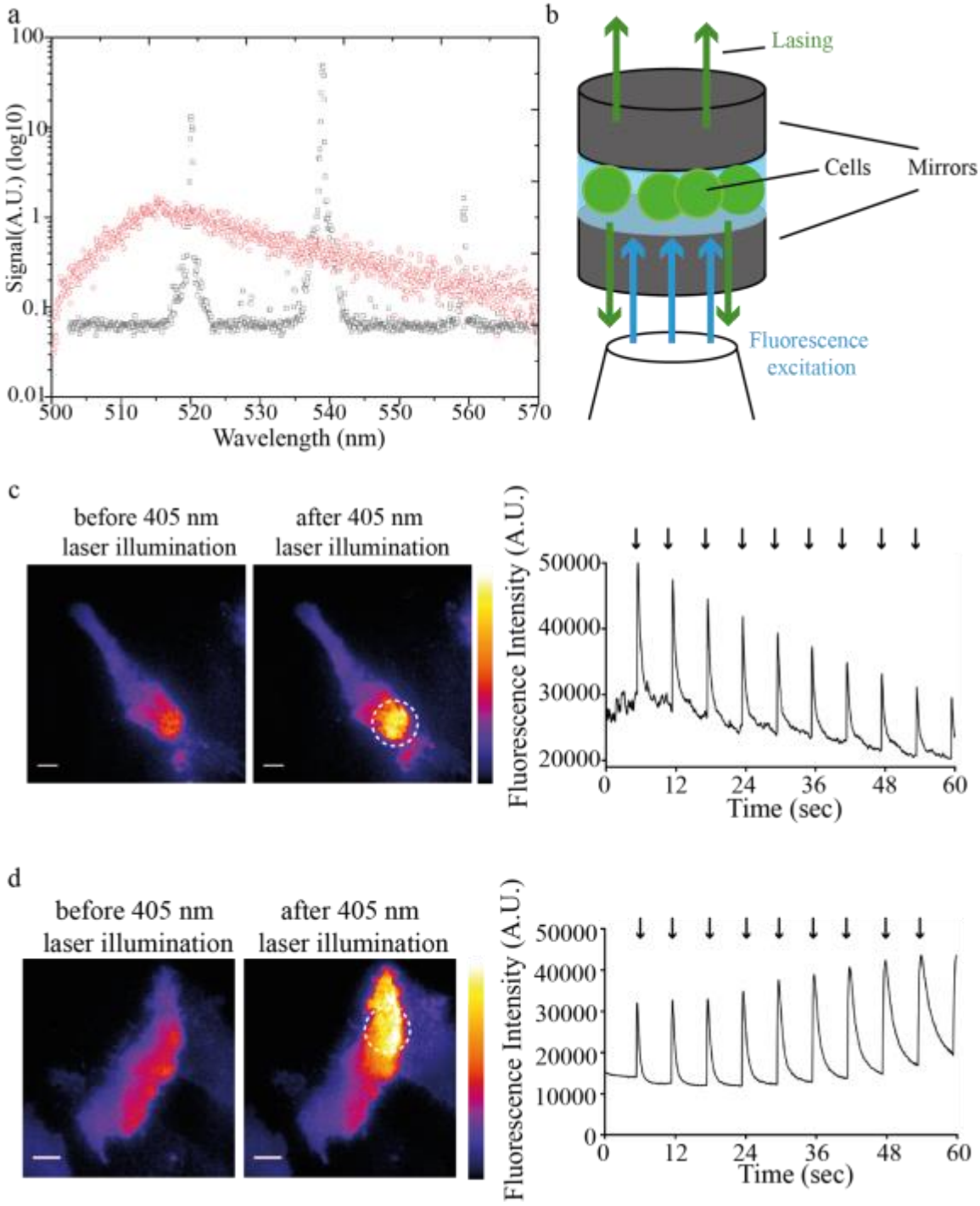

Figure 5.9 Detection of calcium signal changes. (a) Laser emission from individual cell loaded with Calcein AM on the microscope slide and in the laser cavity[212]. (b) Cartoon of cells in the cavity. (c) TIRF images of HEK293 cell loaded with Fluo-3 and CagedCa^{2+}. Fluorescence intensity profile represents the indicated ROI. Arrows indicate the illumination with 405 nm laser. (d) TIRF images of HEK293 cell transfected with GCamp-SNAP-25 and loaded with CagedCa^{2+}. Fluorescence intensity profile represents the indicated ROI. Arrows indicate the illumination with 405 nm laser. Scale bar 5µm.