# Made available by Hasselt University Library in https://documentserver.uhasselt.be

Ensemble and single particle fluorimetric techniques in concerted action to study the diffusion and aggregation of the glycine receptor alpha 3 isoforms in the cell plasma membrane Peer-reviewed author version

NOTELAERS, Kristof; SMISDOM, Nick; Rocha, Susana; JANSSEN, Daniel; Meier, Jochen C.; RIGO, Jean-Michel; Hofkens, Johan & AMELOOT, Marcel (2012) Ensemble and single particle fluorimetric techniques in concerted action to study the diffusion and aggregation of the glycine receptor alpha 3 isoforms in the cell plasma membrane. In: BIOCHIMICA ET BIOPHYSICA ACTA-BIOMEMBRANES, 1818 (12), p. 3131-3140.

DOI: 10.1016/j.bbamem.2012.08.010 Handle: http://hdl.handle.net/1942/14401 Ensemble and single particle fluorimetric techniques in concerted action to study the diffusion and aggregation of the glycine receptor  $\alpha 3$  isoforms in the cell plasma membrane

Kristof Notelaers<sup>\*a,b</sup>, Nick Smisdom<sup>\*a</sup>, Susana Rocha<sup>b</sup>, Daniel Janssen<sup>a</sup>, Jochen C. Meier<sup>c</sup>, Jean-Michel Rigo<sup>a</sup>, Johan Hofkens<sup>b</sup>, Marcel Ameloot<sup>a</sup>

- a Biomedical Research Institute, Hasselt University and School of Life Sciences, transnational University Limburg, Agoralaan gebouw C, 3590 Diepenbeek, Belgium
- b Laboratory for Photochemistry and Spectroscopy, Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200F, B-3001 Heverlee, Belgium
- c RNA Editing and Hyperexcitability Disorders Group, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13092 Berlin, Germany
- \* authors contributed equally

Corresponding author: Marcel Ameloot Agoralaan gebouw C 3590 Diepenbeek Belgium telephone number: +32 11 26 92 33 fax number: +32 11 26 92 99 e-mail: marcel.ameloot@uhasselt.be

#### Abstract

The spatio-temporal membrane behavior of glycine receptors (GlyR) is known to be of influence on receptor homeostasis and functionality. In this work, an elaborate fluorimetric strategy was applied to study the GlyR a3K and L isoforms. Previously established differential clustering, desensitization and synaptic localization of these isoforms implies that membrane behavior is crucial in determining GlyR  $\alpha$ 3 physiology. Therefore diffusion and aggregation of homomeric  $\alpha$ 3 isoform-containing GlyRs were studied in HEK 293 cells. A unique combination of multiple diffraction-limited ensemble average methods and subdiffraction single particle techniques was used in order to achieve an integrated view of receptor properties. Static measurements of aggregation were performed with image correlation spectroscopy (ICS) and, single particle based, direct stochastic optical reconstruction microscopy (dSTORM). Receptor diffusion was measured by means of raster image correlation spectroscopy (RICS), temporal image correlation spectroscopy (TICS), fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT). The results show a significant difference in diffusion coefficient and cluster size between the isoforms. This reveals a positive correlation between desensitization and diffusion and disproves the notion that receptor aggregation is a universal mechanism for accelerated desensitization. The difference in diffusion coefficient between the clustering GlyR a3L and the non-clustering GlyR a3K cannot be explained by normal diffusion. SPT measurements indicate that the  $\alpha$ 3L receptors undergo transient trapping and directed motion, while the GlyR a3K displays mild hindered diffusion. These findings are suggestive of differential molecular interaction of the isoforms after incorporation in the membrane.

# Keywords

glycine receptor, alpha3 isoforms, nanoscopy, single particle, ensemble average, anomalous diffusion

# Abbreviations

$\langle r^2 \rangle$	mean square displacement
$\omega_0$	laser beam radius at 1/e <sup>2</sup> of its maximum intensity
CLSM	confocal laser scanning microscope
D'	Time-dependent diffusion coefficient
D <sub>1-3</sub>	Diffusion coefficient derived from the time lags 1 to 3
DC	dichroic mirror
dSTORM	direct stochastic optical reconstruction microscopy
FRAP	fluorescence recovery after photobleaching
GlyR	glycine receptor
HA	haemagglutinin
HEK 293	human embryonic kidney 293
ICS	image correlation spectroscopy
MWPR	medium without phenol red
PFA	paraformaldehyde
RICS	raster image correlation spectroscopy
ROI	region of interest
SPT	single particle tracking
TICS	temporal image correlation spectroscopy
TIRFM	total internal reflection fluorescence microscope
$t_{lag}$	time lag

# Introduction

The diffusion and aggregation of receptors in the cell membrane have received considerable attention in the last decade.[1-4] In addition to endo- and exocytotic cycling of these membrane proteins, their movement and distribution in the plasma membrane can considerably contribute to the homeostasis of these receptors in the membrane.[5-7] In this way, the cell possesses extra tools to fine-tune receptormediated signaling events.[8-10] An example of this, is surface trapping of neurotransmitter receptors at post-synaptic sites for the facilitation of neurotransmission.[11-14] Hence, a study of the spatiotemporal membrane behavior of neurotransmitter receptors is important in fully comprehending the physiological receptor function.

The transmembranous glycine receptor (GlyR)  $\alpha$ 3 is a neurotransmitter receptor subtype for which membrane properties are implicated in regulating signaling events. Post-transcriptional processing of the *GLRA3* gene transcript [15] gives rise to two isoforms identified as the GlyR  $\alpha$ 3K and  $\alpha$ 3L[16]. The former is the short isoform exhibiting a diffuse membrane staining and fast desensitization kinetics, while the latter contains a 15 amino acid insert (*Figure 1 a*), exhibits a clustered membrane appearance and slow desensitization kinetics.[16,17] An altered expression ratio of these isoforms has been measured in patients with a severe form of temporal lobe epilepsy (TLE).[17-19] A differential synaptic localization of the isoform subunits, has been associated with this observation.[17] In order to improve the understanding of these isoform-related characteristics, this work investigates the spatiotemporal membrane behavior of homomeric[20] (*Figure 1 b*)  $\alpha$ 3K or  $\alpha$ 3L GlyRs on different time and spatial scales.

The GlyR α3 diffusion and aggregation were studied by means of both ensemble average and single particle fluorimetric techniques, either static or dynamic. Earlier work combining fluorescence recovery after photobleaching (FRAP)[21,22] and single particle tracking (SPT)[23-27] measurements for characterization of protein diffusion was inspirational for our fluorimetric approach.[28-30] In this way an integrated view of receptor properties is obtained and possible technical bias in the interpretation is reduced. However both the confocal laser scanning microscope (CLSM)[31,32] and total internal reflection fluorescence microscope (TIRFM)[33-35], used respectively for FRAP and SPT, provide several more techniques for receptor characterization, without requiring drastic changes to the set-up. In this work the CLSM was used not only for FRAP, but also for image correlation spectroscopy (ICS)[36,37] and raster image correlation spectroscopy (RICS)[38,39]. The range of techniques applied on the TIRFM was extended beyond SPT, with temporal image correlation spectroscopy (TICS)[40,41] and single particle based localization microscopy[42-46], in this case direct stochastic optical reconstruction microscopy (dSTORM)[47-49].

The aggregation state of both  $\alpha 3$  isoforms was investigated by ICS and dSTORM. ICS has the advantage that it can be applied on a commercial CLSM and that it can determine the aggregation state

of membrane proteins with very low detection limits. However, ICS is diffraction-limited and does not allow for the direct estimation of the cluster size. This hurdle was overcome by dSTORM, which generates a subdiffraction image. Since the biological samples of both techniques are identical, except for the addition of a reducing agent to the measuring solution, the extra workload to apply both techniques is minimal once they are operational. The diffusion of the receptors was studied at various length and time scales using, in order of decreasing scale, FRAP (>1 µm, seconds to minutes), TICS (diffraction limited, ms to s), RICS (diffraction limited, µs to s) and SPT (subdiffraction technique, ms to s). In addition to the different scales they cover, two fundamentally different types of results are obtained: FRAP, TICS and RICS return all an ensemble averaged result, while SPT generates individual information for every tracked particle. For all fluorescence based techniques employed, an organic fluorophore coupled to a primary antibody was used for receptor labeling, allowing for the use of similarly sized labels for all techniques. Furthermore all dynamic measurements were carried out at 37°C, in order to mimic the effect of body temperature on receptor movement and membrane viscosity.

#### **Materials and Methods**

#### **Cell culture**

Human embryonic kidney 293 cells (HEK 293, kindly provided by Dr. R. Koninckx, Jessa Hospital, Hasselt, Belgium) were maintained at 37°C in a humidified incubator at 5% CO<sub>2</sub> in Dulbecco's modified eagle's medium (Ref. 41966, Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany) and a penicillin (100 IU/ml)-streptomycin (100  $\mu$ g/ml) mixture (Invitrogen, Merelbeke, Belgium). The cells used for microscopic observation were plated two days before the experiment in 8-well Lab-Tek<sup>TM</sup> II chambered coverglass (Nalge Nunc International, Rochester, NY, USA) seeded at a density of 20,000 cells per well in transfection medium. This is Dulbecco's modified eagle's medium (Ref. 41965, Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum but without the penicillin/streptomycin mixture.

#### Transfection

After overnight incubation, the cells were transfected using calcium phosphate co-precipitation with plasmids encoding for the haemagglutinin (HA)-tagged (*Figure 1 a*) splice variants  $\alpha$ 3K and  $\alpha$ 3L of the mouse GlyR  $\alpha$ 3.[17] The HA-tag was located in the extracellular N-terminal domain between amino acids 35 and 36.[17] The plasmids were diluted in 250 mM CaCl<sub>2</sub> buffer at a concentration of 20 ng/µl and an equal amount of HEPES buffered saline solution (HBS, pH 7.05) was added dropwise. After incubation of 15 minutes, this mixture was added to the culture medium of the cells. All cell recipients used in the various experiments received a final amount of DNA of 0.21 µg/cm<sup>2</sup>. After 6 hours, the transfection medium was replaced with transfection medium supplemented with 200 nM strychnine. All measurements occurred within 24 to 36 hours after transfection.

# Labeling of exogenous GlyRs

A chicken polyclonal anti-HA antibody (Bethyl lab Inc, Montgomery, TX, USA) was used to stain the HA-tagged GlyRs. This antibody was directly labeled with Alexa Fluor 488 (Alexa 488) or Alexa Fluor 647 (Alexa 647) using a commercial Alexa Fluor 488/647 tetrafluorophenyl labeling kit (Molecular Probes/Invitrogen) according to the manufacturer's protocol. Two vials of reactive fluorochrome were used to increase the degree of labeling. The degree of labeling was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at 3.9 (Alexa 488) and 4.4 (Alexa 647) labels per antibody. Cells transfected with the GlyR  $\alpha$ 3 were rinsed twice with HEPES-buffered DMEM without phenol red (Ref. 21063, Gibco), abbreviated as MWPR (medium without phenol red), and incubated for 10 minutes at 37°C with a 4 µg/ml (Alexa 488) and 2 µg/ml (Alexa 647) antibody solution. Afterwards, cells were rinsed three times with MWPR and used for live cell imaging (FRAP, RICS, TICS, SPT) or fixed for 10 minutes using 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (ICS, dSTORM).

#### **Image correlation spectroscopy**

Bottom membrane images of fixed cells were acquired using a Zeiss LSM 510 META (Carl Zeiss, Jena, Germany) CLSM on an inverted epifluorescence Axiovert 200M motorized frame equipped with an αPLAN-APOCHROMAT 100x/1.46 objective (Carl Zeiss). 150 fs pulsed laser light of a Titanium:Sapphire laser (Mai Tai, Spectra-Physics, Irvine, CA, USA) tuned at an output wavelength of 930 nm (1.4 W output power) with 1.5 mW on the stage was used to excite Alexa 488. The emission light was detected using non-descanned detection: the fluorescence was directed using a dichroic mirror (DC) FT685, a short-pass KP685 and a band-pass BP495-545 towards a photomultiplier tube (PMT; Hamamatsu 7422, Herrsching am Ammersee, Germany). This PMT was connected to an SPC830 card (Becker and Hickl, Berlin, Germany) synchronized by the scan pulses from the CLSM. Data were acquired in FIFO imaging mode. The resulting images have a 512 by 512 resolution, a pixel size of 22 nm, and a pixel dwell time of 51.2 μs. Data were analyzed using in-house developed routines written in a MATLAB® environment (R2010b, The Mathworks, Gouda, The Netherlands) according to a published protocol for ultrasensitive detection.[37]

# Direct stochastic optical reconstruction microscopy

Transfected cells were stained with Alexa 488 and fixed prior to incubation with a buffered solution containing 100 mM cysteamine (Sigma-Aldrich). dSTORM was carried out on these cells at room temperature using an inverted epi-fluorescence microscope (Olympus IX71 frame S1F-3, Olympus Optical, Tokyo, Japan) equipped with a PlanApo 60x oil objective(NA 1.45; Olympus Optical). The beams of a 100 mW 488 nm (Excelsior 488, Spectra-Physics) and a 100 mW 405 nm (Cube Coherent) diode laser were consecutively selected by mechanical shutters during respectively 50 and 40 ms at a repetition rate of 10 Hz. This light was directed by the DC (z405/491rdc; Chroma, Bellow Falls, VT, USA) towards the objective in off-axis mode to obtain total internal reflection. Excitation light was removed using the DC and a HQ500LP emission filter (Chroma). The 488 nm excitation light was used to excite the Alexa 488 and to obtain a suitable off switching rate. The on switching rate was controlled by the 405 nm laser light with 40 ms exposure time in the overall sequence.[50] The images were recorded at 10 Hz using an EM-CCD camera (ImageEM, Hamamatsu) with 50 ms integration time, a resolution of  $512 \times 512$  and an image pixel size of 80 nm. The camera was synchronized with the exposure time of the 488 nm laser light, reducing additional background introduced by the 405 nm light exposure. Using home-developed software the particle positions were determined and mapped out on a 20 nm pixel grid corresponding to the localization precision[51,52]. Multi-distance spatial clustering analysis was performed on the obtained point maps using Ripley's H-function[53]. This function is derived from Ripley's K-function[54,55], but represents clustering and dispersions by means of positive and negative values respectively.

# Fluorescence recovery after photobleaching

FRAP measurements were performed using a Zeiss LSM 510 META CLSM (Carl Zeiss) on an epifluorescence Axiovert 200M motorized frame. The Alexa 488 was excited with the 488 nm line (selected by a  $488 \pm 10$  nm interference-base laser cleanup filter) of the 30 mW air-cooled argon ion laser (5.5 A tube-current) under the control of an AOTF (set at 0.8% transmission). The excitation light was directed to the sample via a DC (HFT 488) and a Plan-Neofluar 40x/1.3 oil immersion objective (Carl Zeiss). The fluorescence light was detected in descanned mode through the DC and a long-pass LP505 emission filter to the PMT. The pinhole size was set to 3 Airy units and the image size was typically set to  $512 \times 512$  pixels with a pixel size of 63 nm. The interval between the start of subsequent frames was determined as  $1/3 \tau_D$ . Each time series typically comprised 2 pre-bleach frames and 18 recovery frames. Cells were kept at 37°C by means of a small stage incubator and an objective heater (Pecon, Erbach, Germany). Resulting time series were analyzed using custom written routines in a MATLAB environment based on published software components.[22] A new approach was introduced to select a reference region that comprises the bleach region of interest (ROI) and its perimeter in which the fluorescence can be assumed to be constant after bleaching occurred. This approach allows extraction of the recovery curve without user biased selection of a reference region and finds its origin in a procedure suggested by Phair [56,57] (suppl. mat. 1) Two FRAP models are used in the analysis step: the uniform disk model[58] and the generalized disk model[22]. The latter model was implemented with the instrumental resolution factor fixed at the estimated value of 0.07  $\mu m^2$ .

#### **Raster image correlation spectroscopy**

The RICS measurements were performed with the identical setup as used for ICS. A small stage incubator and an objective heater (Pecon) were used to keep the cells at 37 °C. Besides the  $\alpha$ Plan-Apochromat 100x/1.46 objective (Carl Zeiss), also a LD C-Apochromat 40x/1.1 W Korr UV-VIS-IR objective (Carl Zeiss) was used. Pixel sizes were respectively 22.1 nm and 54.8 nm and image resolution was 512 × 512. Pixel dwell times ( $\tau_P$ ) of 163.9 µs, 102 µs, 51.8 µs, 25.6 µs and 6.4 µs were used. This range of scan speeds was used to explore possible fast motion of the receptors. The associated line times can be calculated as  $1200\tau_P n_{tracks}$ , where  $n_{tracks}$  is the number of times each line is sequentially scanned before proceeding to the next line. These multiple scanning of each line was applied to increase the line time to further expand the dynamic range of the technique. Image analysis was performed in the MATLAB environment as published elsewhere.[59]

#### **Temporal image correlation spectroscopy**

Transfected cells were stained as described and kept at 37°C through the use of a commercial stage incubator (Pecon GmbH, Erbach, Germany). Cargille type 37 (Cargille Labs, Cedar Grove, NJ, USA) was used as immersion oil. All images were acquired using the setup as described for dSTORM, but

without the 405 nm laser light. Two neutral density filters (optical density 2.5 and 0.1) were introduced to minimize photobleaching of Alexa 488 as much as possible. To reduce the photobleaching even further, a shutter in the beam path, which also triggered the camera, was used to block the laser light between consecutive images. The exposure time of the camera was kept constant at 50 ms and the EM gain was set at 149. Images were acquired at various acquisition rates ranging from 50 ms to 5 s. Data analysis was performed using custom written software in the MATLAB environment according to published approaches.[40,60] A correction for photobleaching was applied as described.[40]

#### Single particle tracking

The SPT measurements were carried out on the set-up that also accommodated the dSTORM and TICS measurements.[61] Alexa 647 was used with excitation from a 60 mW 642 nm diode laser (Excelsior 642, Spectra-Physics), a z647rdc DC and a 665LP emission filter (Chroma). The exposure time of the camera was kept constant at 50 ms and the EM gain was set at 610. Bottom membrane images were acquired at 10 Hz with shuttered exposure to minimize photobleaching. Live cells were kept at 37°C through the use of a commercial stage incubator (Pecon) and Cargille type 37 was used as immersion oil. Particle detection and localization, trajectory construction and calculation of the mean square displacements ( $\langle r^2 \rangle$ ) for each time lag ( $t_{lag}$ ) were done by in-house developed MATLAB® routines. Only trajectories with a minimum of 16 time steps were considered. The localization precision (σ), determined by tracking of Alexa 647 labeled GlyRs at 37°C in cell membranes fixed for 10 minutes with 4% PFA, was kept fixed. For short time range diffusion, the diffusion coefficient was determined by fitting the first three time lags (D<sub>1-3</sub>) of trajectories with the linear function  $\langle r^2 \rangle = 4Dt_{lag} + 4\sigma^2$ .[62] For long time range diffusion, fitting of the first quarter[62] of the displacement data was done using the non-linear function  $\langle r^2 \rangle = 4D' t_{lag}^{\alpha} + 4\sigma^2$  [30,63], with D' and  $\alpha$  as freely adjustable fitting parameters. The  $\alpha$ -exponent allows for a time-dependent diffusion coefficient (D'), which improved fitting significantly. Tracks were considered immobile if the  $\langle r^2 \rangle_{t_{log}} < 4\sigma^2$  for any time lags in the fit or if  $\langle r^2 \rangle_{t_{log}} = 1 > \langle r^2 \rangle_{t_{log}} > 1$ .

# Results

Each experimental technique was applied to two separate expression systems. Each system comprised the expression of a different  $\alpha 3$  (K or L) subunit isoform in HEK 293 cells. For both isoforms individual expression of the subunit lead to the formation of functional transmembrane receptors. This was verified by analyzing protein expression with western blot and measuring glycine-evoked membrane currents using whole-cell patch-clamp (suppl. mat. 2).

# GlyR α3 aggregation state

The membrane aggregation state of the GlyR  $\alpha$ 3 isoforms in fixed samples was studied by using ICS and dSTORM. ICS analysis was carried out on regions of the bottom membrane with an apparent homogeneous distribution of fluorescent entities (*Figure 2*). The average brightness (counts/s per entity) was determined by combining the measured average density of fluorescent entities together with the associated average fluorescence intensity of the image (*Table 1*).[37] Using this analysis, the fluorescent entities of GlyR  $\alpha$ 3L are found to be approximately 8 times (7.5 ± 0.4) brighter as compared to their GlyR  $\alpha$ 3K counterparts. Assuming that the brightness of the fluorescent labels is independent of the aggregation state of the receptor and that each fluorescent entity of GlyR  $\alpha$ 3K corresponds to an individual receptor, it can be concluded that each fluorescent entity of GlyR  $\alpha$ 3L clusters is below the diffraction limit as the observed radial beam radius  $\omega_0$  is essentially constant (Table 1).

dSTORM experiments yield point accumulation images representing a map of GlyR  $\alpha$ 3 locations in the bottom membrane (*Figure 3 a,b*). Analysis of these point locations by Ripley's H-function indicates a clear deviation from random distribution for the GlyR  $\alpha$ 3L, and this in contrast to the GlyR  $\alpha$ 3K. The GlyR  $\alpha$ 3L point distribution yields positive values for the H-function, up to 1 µm and with the maximum of the H-function at 280 nm. For the GlyR  $\alpha$ 3K the maximum of the H-function is at a distance of  $\approx$  20 nm, which is of the same order as the localization precision.

# Study of GlyR α3 diffusion using ensemble techniques

FRAP experiments analyzed using the uniform disk model and the new reference method yielded a diffusion coefficient of  $0.15 \pm 0.01 \ \mu m^2/s$  with a mobile fraction of  $0.93 \pm 0.04$  for the GlyR  $\alpha 3K$  (*Figure 4 a*). Variations in ROI size did not reveal changes in diffusion coefficient (*Figure 4 c*) nor in mobile fraction (data not shown). Unfortunately, due to the low density of the bright clusters from GlyR  $\alpha 3L$ , applying FRAP to this isoform was unsuccessful (*Figure 4 b*). Movement of these bright clusters in and out of the bleach region dramatically affects the recovery curve and renders it unsuitable for further analysis.

Analysis of diffusion measurements carried out with RICS reveals a difference in diffusion coefficient of more than an order of magnitude between the GlyR  $\alpha$ 3K and  $\alpha$ 3L (respectively 0.11 ± 0.02  $\mu$ m<sup>2</sup>/s and 0.008 ± 0.002  $\mu$ m<sup>2</sup>/s). Coarse spatial mapping of receptor diffusion in the bottom membrane, displays variations that are relatively small compared to the inter-isoform difference (*Figure 5 a, b*). Application of several scan speeds confirmed that no faster moving fraction of both types of receptors existed.

TICS experiments performed on the GlyR  $\alpha$ 3K and  $\alpha$ 3L revealed that both splice variants contain two species of receptor diffusion (*Figure 6*). Due to bleaching and the limitations of the photobleaching correction (suppl. mat. 3), only the diffusion of the fastest species could be reliably characterized. The average values across the experiments for these species are 0.16 ± 0.07 µm<sup>2</sup>/s for the GlyR  $\alpha$ 3K and 0.021 ± 0.009 µm<sup>2</sup>/s for the GlyR  $\alpha$ 3L. In summary, all ensemble techniques yield similar diffusion coefficients for each individual isoform (*Table 2*).

#### Study of the GlyR $\alpha$ 3 diffusion using single particle tracking

The diffusing GlyR  $\alpha$ 3 isoforms were monitored by tracking individual fluorescent features in the bottom membrane (*Figure 7 a, b*). The obtained distribution for short time range D<sub>1-3</sub> is shifted towards faster diffusion for the GlyR  $\alpha$ 3K compared to the GlyR  $\alpha$ 3L, with respective averages of 0.13 µm<sup>2</sup>/s and 0.044 µm<sup>2</sup>/s (*Figure 7 c*). For longer time ranges, a time-dependent diffusion coefficient better suited the data. This implied that the average  $\alpha$ -exponents were indicative of anomalous diffusion ( $\alpha \neq 1$ ), equaling 0.87 for the GlyR  $\alpha$ 3K and 0.73 for the GlyR  $\alpha$ 3L. The  $\alpha$ -exponents exhibit a broad distribution due to imperfect sampling[29]. Nevertheless a clear distinction can be made between the GlyR  $\alpha$ 3K and  $\alpha$ 3L. The distribution of the former has a zero skewness centered on normal diffusion ( $\alpha = 1$ ), yet contains more weight on anomalous subdiffusion ( $\alpha < 1$ ) (*Figure 7 d*). The distribution of the latter has positive non-zero skewness, with the center on anomalous subdiffusion and with a long tail to the right suggesting the presence of anomalous superdiffusion ( $\alpha > 1$ ) (*Figure 7 d*). For both receptor types a fraction of the analyzed trajectories was designated as immobile within the time-dependent diffusion model approach. This concerned 5% of the trajectories for the GlyR  $\alpha$ 3K and 15% for the GlyR  $\alpha$ 3L (*Table 3*).

Upon inspection of single trajectories, it can be ascertained that transient trapping is present for particles displaying tracks with anomalous subdiffusion (*Figure 8 a, b*). This was observed for both  $\alpha$ 3K and  $\alpha$ 3L receptors. On the other hand, tracks corresponding with anomalous superdiffusion reflect clear directed motion combined with local trapping (*Figure 8 c, d*). The latter type of complex particle motion was only observed for the GlyR  $\alpha$ 3L.

# Discussion

In this study, differential diffusion and aggregation of the homomeric GlyR  $\alpha$ 3 related to the isoform composition were investigated. The difference in aggregation states of the GlyR isoforms  $\alpha$ 3K and  $\alpha$ 3L was confirmed using ICS and dSTORM. The differences in receptor diffusion were studied with a variety fluorimetric techniques. Further discussion pertains to the integrated view of receptor diffusion achieved by combining ensemble and single particle fluorimetric techniques. Finally, the results are placed in a physiological context and the perspectives for further study of the GlyR  $\alpha$ 3 are outlined.

#### The GlyR $\alpha$ 3 isoforms differ in aggregation state

The difference between the diffuse staining pattern of GlyR  $\alpha$ 3K and the punctate staining pattern of GlyR  $\alpha$ 3L is obvious, even through regular microscopic observation. This differential staining pattern reflects the different aggregation state of both receptor isoforms. Eichler *et al.* estimated the cluster size of GlyR  $\alpha$ 3L in transfected primary hippocampal neurons, but the number of receptors per cluster could not be determined.[17] In order to estimate this number and to enable the detection of even smaller differences in aggregation state, the application of ICS and dSTORM to the current expression systems was explored. Both methods start from a static snapshot of the cellular model obtained through chemical fixation.

The ICS results revealed that each GlyR  $\alpha$ 3L cluster comprised on average 8 labeled receptors assuming the exclusive presence of individual receptors for GlyR  $\alpha$ 3K and a constant brightness per receptor. When aggregation reduces this brightness, as could well occur by steric hindrance of bound antibody, 8 will be a lower limit. Furthermore, the size of the detected GlyR  $\alpha$ 3L clusters was still below the diffraction limit, as indicated by the lack of increment of the average observation profile radius  $\omega_0$  retrieved in ICS (Table 1). This is consistent with the estimated number of labeled receptors in combination with the size of the GlyR, which should be close to the published 8.5 nm radial diameter of the structurally related nicotinic acetylcholine receptor.[64,65]

In addition to the diffraction-limited ICS, the super-resolution method dSTORM was applied to obtain sub-diffraction resolution images of the receptors. Analysis of these data using Ripley's H-function indicates a clear difference in aggregation state between the isoforms. While the GlyR  $\alpha$ 3L displays a non-uniform clustering distribution, any clustering seen with the GlyR  $\alpha$ 3K is minimal and barely exceeds artifacts generated by the repeated localization of identical particles.[66] The maximum of Ripley's H-function cannot be used as a rigorous estimate of the mean cluster size as this maximum depends on the cluster separation.[67] Also, a distribution of cluster sizes cannot be excluded and the higher number of detected events for larger clusters can introduce a bias.

#### GlyR $\alpha$ 3 diffusion studied by ensemble techniques

The diffusion of GlyR  $\alpha$ 3K was successfully studied by FRAP. Two models for free diffusion, which differ in their assumption of the shape of the bleaching beam, were cautiously applied. It is known that a model for free diffusion might yield incorrect results in presence of anomalous diffusion while apparently being correct based on the quality of the fit.[68] However, the low immobile fraction observed by both models in the experiments reflects the suitability of the free diffusion. Furthermore, for receptors undergoing anomalous diffusion, the immobile fraction would be much higher upon analysis with a constant diffusion coefficient. Finally, the lack of dependency of both D and the immobile fraction of anomalous diffusion. The results of both models did not differ significantly.

Unfortunately, GlyR  $\alpha$ 3L could not be studied by FRAP due to the presence of clusters. Movement of clusters in and out the ROI dramatically affects the fluorescence intensity inside the ROI. A similar effect is seen when the concentration of fluorophores is too low.[69]

While RICS in a biological context is mainly used to measure the diffusion of proteins in the cytoplasm [38,70], this technique has been previously used by us to characterize diffusion of membrane components[59,71]. The low diffusion coefficients of membrane proteins force the application of low scan speeds near the limit of the capabilities of our CLSM set-up. Coarse spatial mapping achieved with RICS revealed a limited spatial dependency of the diffusion coefficient for each individual isoform over the cell surface.[39,59] In contrast to FRAP, RICS was able to measure the diffusion coefficient of both isoforms, with a good agreement for both techniques for the K isoform. The difference in magnitude of the obtained diffusion coefficients of both isoforms is larger than expected based on their difference in aggregation state estimated with ICS and dSTORM. This provides evidence for an influence, not inherent to the receptors, such as the membrane constitution or submembranous structures.

TICS was originally applied on a CLSM[40], but the available frame rate would not allow to resolve the large, fast moving fractions of the proteins. Therefore, we used a TIRFM with a frame rate optimized for the time scale of the expected kinetics. From simulated data (suppl. mat. 3), it is shown that for the observed bleaching rate, the experimental data can be corrected appropriately so that reliable estimates for the diffusion coefficient of the fastest species can be obtained. The use of TICS revealed the presence of an apparent slower diffusing species, but due to the bleaching rate this information could not unambiguously be interpreted.

#### SPT reveals anomalous diffusion in GlyR $\alpha$ 3 diffusion

The short time range D<sub>1-3</sub> derived from SPT confirm faster diffusion for the GlyR α3K in comparison to the GlyR  $\alpha$ 3L. This could be explained by cluster formation of  $\alpha$ 3L receptors, as seen by ICS and dSTORM, resulting in large complexes exhibiting slow but normal membrane diffusion. However, the observation that for a longer time range the diffusion coefficient becomes time-dependent, suggests that a larger hydrodynamic radius due to clustering of the GlyR  $\alpha$ 3L cannot be the only determining factor. The average of the  $\alpha$ -exponents ( $\alpha < 1$ ) indicates that both receptor isoforms undergo subdiffusion. The fact that this is more pronounced for the GlyR a3L, which also displays a larger immobile fraction and a higher degree of clustering, supports the idea of local transient trapping of receptors in small regions. [72,73] This can be qualitatively confirmed by visual inspection of the GlyR  $\alpha$ 3L tracks. A lower degree of subdiffusion for the GlyR  $\alpha$ 3K could indicate that these receptors are transiently trapped in larger regions. However, the size and the distribution of these regions has to be consistent with the nearly uniform distribution of the GlyR  $\alpha$ 3K observed with dSTORM. An alternative assumption is that the  $\alpha 3K$  isoform is less prone to trapping and therefore resides less in the trapped state. A higher degree of interaction determining the motion of GlyR  $\alpha$ 3L is corroborated by the presence of anomalous superdiffusion [74] in the receptor motion. The combination of trapping and superdiffusion, as found in the trajectories of the GlyR  $\alpha$ 3L, is likely to represent receptors actively being transported from, to or between different locations in the membrane.

# An integrated view of receptor diffusion

Given the spatio-temporal properties of the GlyR a3 diffusion, SPT allowed for the most detailed characterization. However, the possible presence of bias inherent to SPT or the application of SPT to live cells warrants a combination with ensemble techniques. A first aspect is that highly mobile particles are more difficult to track compared to their slower moving counterparts, meaning a possible bias towards types of subdiffusion can be present. Upon observation of subdiffusion, as for the GlyR  $\alpha$ 3, a comparison with FRAP experiments is of importance, to estimate the relevance on the cellular level. In the instance of the GlyR  $\alpha$ 3K this revealed a minimal impact of the subdiffusion measured by SPT, on large scale receptor motion measured with FRAP.[68] Second, due to the bleaching of the particles, a bias towards fresh molecules diffusing into the bottom membrane from outside the measurement plane can arise. A comparison of the diffusion parameters measured with techniques requiring minimal measurement bleaching, such as TICS and FRAP, can resolve whether representative fractions are measured. In our case a good agreement between diffusion coefficients from single molecule tracking  $(D_{1-3})$  and ensemble techniques was found. Moreover, a good agreement between the immobile fractions measured with FRAP and SPT was measured. Furthermore, coarse spatial mapping with RICS confirmed homogeneous diffusion patterns on the cellular level. Third, in choosing cells for SPT measurements, a selection bias can occur towards cells yielding the highest fluorescence signal or containing bright fluorescent entities. Cells showing an overall high level of protein expression after transfection are usually unhealthy[75] and the presence of bright fluorescent entities can bias particle detection parameters. Again, comparing the results obtained with SPT to those obtained with ensemble techniques, where cells are selected based on the ensemble fluorescence signal, decreases the risk of biased sampling. Exemplary is the detection of multiple species with TICS, which confirms that the complex GlyR  $\alpha$ 3 motion found with SPT is not a technique related artifact.

#### Perspectives for the GlyR a3

The diffusion coefficients reported here for the GlyR  $\alpha 3$  are within the range expected for proteins diffusing in the cell membrane. [76,77] However, experimental parameters such as the applied cell line, measurement temperature and time of measurement, given their influence on membrane viscosity, thermal agitation, expression level and molecular interactions, are key factors when comparing protein diffusion. Variations in these parameters hamper a comparison with reported values for other GlyR subtypes.[78-80] Nevertheless the results can be placed in a biological context. Receptor diffusion plays an essential role in neuronal function as it allows for renewal of receptors in the desensitized state and regulation of postsynaptic receptor pool size. [12,81] Homomeric GlyR  $\alpha$ 3K channels were shown to contribute to tonic inhibition of neuronal excitability, which requires receptors in the nondesensitized state. [14,18] Compared with  $\alpha$ 3L, GlyR  $\alpha$ 3K desensitizes more rapidly [16] and, therefore, its faster diffusion makes sense as it increases the probability of receptor cycling between endocytic and cell plasma membrane compartments, which will facilitate receptor renewal.[12] Furthermore, this study adds to the understanding of receptor desensitization mechanisms. In fact, it was shown that increased GlyR  $\alpha 1$  receptor density in gephyrin-dependent clusters facilitates desensitization, whereas in case of GlyR a3L just the opposite was observed.[9,16] Thus, GlyR clustering is not a universal mechanism of desensitization but also involves receptor conformation depending on primary structure or receptor associated proteins other than gephyrin.[82,83]

A next step towards understanding the role of RNA splicing in GlyR  $\alpha$ 3 function will be the identification of proteins that interact with the RNA splice insert TEAFALEKFYRFSDT located in the large cytosolic loop between transmembrane domains 3 and 4 of GlyR  $\alpha$ 3L. A similar fluorimetric strategy as formulated here can be used to explore different biological settings. Pharmacological breakdown of the cytoskeleton has been shown to influence GlyR dynamics in cultured neurons.[79,80] Another option is depleting membrane cholesterol, thereby influencing the different types of lipid rafts which have been reported in HeLa cells.[84] Disturbance of lipid rafts can lead to dissociation of raft induced protein aggregation and altered protein function.[85,86] An alternative approach for studying GlyR  $\alpha$ 3 interactions involves site-directed mutagenesis of the insert region. This has previously been applied for studying the regulatory capabilities of the splice insert on channel

gating and domain structure.[87] By probing all these conditions, mechanisms determining the GlyR  $\alpha$ 3 behavior can be better understood.

An appropriate exploratory strategy for examining any transmembrane protein should maximize efficiency in screening multiple conditions and minimize the risk of overlooking any changes in receptor behavior. Therefore labor intensity, applicability and spatio-temporal sampling should be considered when developing a screening approach. This report offers a strong foundation for making these considerations.

## Acknowledgements

We would like to thank Ir. Ben De Clercq for providing us with the proper settings of the Becker and Hickl detection system. GlyR expression constructs were obtained thanks to funding by the Helmholtz Association (VH-NG-246 to J.C.M.). S.R. and J.H. acknowledge financial support of the "Fonds voor Wetenschappelijk Onderzoek FWO" (grants G.0402.09, G.0413.10, G.0697.11), the K. U. Leuven Research Fund (GOA 2011/03), the Flemish government (long-term structural funding: Methusalem funding CASAS METH/08/04). S.R., J.H. and M.A. thank the Federal Science Policy of Belgium (IAP-VI/27). M.A. recognizes the Province of Limburg (Belgium) for the financial support within the tUL IMPULS FASE II program allowing for the upgrading the laser source used in this work. The support by the FWO-onderzoeksgemeenschap "Scanning and Wide Field Microscopy of (Bio)-organic Systems" is gratefully acknowledged by J.H. and M.A..

# Tables

Table 1: ICS analysis performed on fixed cells e	xpressing either GlyR	$\alpha 3K$ or GlyR (	a3L. Particle	density and	brightness
were corrected for background intensity and non-s	pecific binding of the a	ntibody.			

Splice variant <sup>a</sup>	Density (entities/µm <sup>2</sup> )	Brightness (counts/s entity)	ω <sub>0</sub> (nm)	
GlyR a3K	$5.9\pm0.8^{\text{b}}$	$0.28\pm0.04$	$43{\times}10\pm8{\times}10$	
GlyR a3L	$1.3 \pm 0.3$	$2.1\pm0.4$	$40 {\times} 10 \pm 2 {\times} 10$	
a For each isoform 9 c	ells were measured			

b Errors are reported as standard error of the mean

Table 2: The average diffusion coefficient (in µm<sup>2</sup>/s) as obtained per ensemble microfluorimetric technique.

Splice variant	FRAP	RICS	TICS
GlyR α3K <sup>a</sup>	$0.15\pm0.01^{\rm c}$	$0.11\pm0.02$	$0.16\pm0.07$
GlyR $\alpha 3L^{b}$	Not Feasible	$0.008 \pm 0.002$	$0.021\pm0.009$
a Cells analyzed per techno	aiue, FRAP: 31, RICS: 6, TICS: 6		

b

Cells analyzed per technique. FRAP: 0, RICS: 6, TICS: 6 Errors are represented as standard error of the mean

с

Table 3: The average short time range  $D_{1.3}$  (in  $\mu$ m<sup>2</sup>/s) are reported for both isoforms. The averages of the free fit parameters (D', a-exponents) from the non-linear fit of diffusion at longer time range are represented together with the immobile fraction.

SPT	Short time range	Long time range		
Splice variant	$\langle D_{ ext{1-3}}  angle$	$\langle D'  angle$	a-exponent	Immobile fraction <sup>d</sup>
GlyR $\alpha 3K^a$	$0.129\pm0.002^{\rm c}$	$0.133\pm0.003$	$0.866 \pm 0.008$	5%
GlyR $\alpha 3L^b$	$0.0436 \pm 0.0008$	$0.042\pm0.001$	$0.736\pm0.006$	15%

Number of cells measured: 7, number of trajectories analyzed: 1629 а

Number of cells measured: 9, number of trajectories analyzed: 4291 b

Errors are represented as standard error of the mean с

d See materials and methods for criteria

# **Figure captions**

**Figure 1:** Schematic representation of the glycine receptor structure. a) General structure of a GlyR subunit showing the four transmembrane  $\alpha$  helices (TM1 – TM4) and the large, extracellular N-terminal domain. The location of the HA-tag used in this study is indicated, as well as the region of the 15 extra amino acids of the L isoform. b) A functional, homomeric GlyR comprises five subunits, with TM2 of each subunit lining the pore.

**Figure 2:** Representative images obtained from respectively GlyR  $\alpha$ 3K and  $\alpha$ 3L in fixed HEK 293 cells are shown by (a) and (b). Both images have the same intensity scale. Qualitative inspection reveals a diffuse staining of the K splice variant and a punctate pattern of GlyR  $\alpha$ 3L. (c) and (d) are the experimentally obtained spatial autocorrelation function (gray shade, facing quadrant removed) of respectively GlyR  $\alpha$ 3K and  $\alpha$ 3L, together with the best fit (colored surface). The plot of the weighted residuals is indicated above the corresponding spatial autocorrelation function.

**Figure 3:** Representative accumulated point map images obtained from respectively the GlyR  $\alpha$ 3K (a) and the GlyR  $\alpha$ 3L (b) in fixed HEK 293 cells using dSTORM. Both images have the same intensity scale. Ripley's multi-distance spatial clustering analysis (c) reveals profound clustering of the GlyR  $\alpha$ 3L compared to the GlyR  $\alpha$ 3K.

**Figure 4:** Representative fluorescence recovery from respectively GlyR  $\alpha$ 3K and  $\alpha$ 3L expressed in HEK 293 cells are shown by (a) and (b). For the GlyR  $\alpha$ 3K, the model properly fit the curve, while fitting the recovery curve of GlyR  $\alpha$ 3L was unsuccessful. Diffusion coefficients for the GlyR  $\alpha$ 3K obtained from 31 cells and analyzed using the uniform disk model are plotted as the average and standard deviation per ROI radius (c). The overall average of the diffusion coefficient is 0.15 ± 0.01  $\mu$ m<sup>2</sup>/s and of the mobile fraction is 0.93 ± 0.04.

**Figure 5:** RICS of live HEK 293 cells expressing GlyR  $\alpha$ 3K (a) and  $\alpha$ 3L (b) allows for a coarse mapping of the mobility of the respective receptors. Each white rectangular box represents an analyzed area with the obtained diffusion coefficient [in  $\mu$ m<sup>2</sup>/s] and standard error reported. In general, there was a difference of over one decade between the diffusion coefficients of both splice variants. In addition, there was also variation of the diffusion coefficient within a single cell, albeit to a lesser extent. An identical color map was applied for both images.

**Figure 6:** The temporal autocorrelation function obtained in TICS analysis of GlyR  $\alpha$ 3K (a) and  $\alpha$ 3L (b) are plotted, together with the 1 species and 2 species fit. The upper panels show the weighted residuals.

**Figure 7:** Representative trajectory plots, shown for the GlyR  $\alpha$ 3K (a) and  $\alpha$ 3L (b). Comparison of the cumulative distributions of short time range D<sub>1-3</sub> shows faster overall diffusion for the GlyR  $\alpha$ 3K compared to the GlyR  $\alpha$ 3L (c). The distribution of  $\alpha$ -exponents indicates anomalous subdiffusion in both receptor populations (d). For the GlyR  $\alpha$ 3L, a subset of tracks also displays anomalous superdiffusion (arrow). (bin = number of elements in bin, tot = total elements in distribution)

**Figure 8:** To interpret the deviation from normal diffusion, representative tracks from particles with anomalous  $\alpha$ -exponents are shown (a, c). Particles with an  $\alpha$ -exponent < 1 exhibit diffusion with transient trapping (a), the localization precision is also represented (dashed line, b). Particles with an  $\alpha$ -exponent > 1 show a heterogeneous displacement pattern (c), where the proportion of superdiffusion outweighs the subdiffusion (d).

# References

- [1] L. Groc, L. Bard, D. Choquet, Surface trafficking of N-methyl-D-aspartate receptors: physiological and pathological perspectives, Neuroscience, 158 (2009) 4-18.
- [2] F.J. Barrantes, Cholesterol effects on nicotinic acetylcholine receptor, J. Neurochem., 103 Suppl 1 (2007) 72-80.
- [3] I. Chung, R. Akita, R. Vandlen, D. Toomre, J. Schlessinger, I. Mellman, Spatial control of EGF receptor activation by reversible dimerization on living cells, Nature, 464 (2010) 783-787.
- [4] Z.Q. Xu, X. Zhang, L. Scott, Regulation of G protein-coupled receptor trafficking, Acta Physiol. (Oxf), 190 (2007) 39-45.
- [5] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science, 327 (2010) 46-50.
- [6] K. Ritchie, R. Iino, T. Fujiwara, K. Murase, A. Kusumi, The fence and picket structure of the plasma membrane of live cells as revealed by single molecule techniques (Review), Mol. Membr. Biol., 20 (2003) 13-18.
- [7] Y. Sako, A. Kusumi, Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis, J. Cell. Biol., 125 (1994) 1251-1264.
- [8] C. Bedet, J.C. Bruusgaard, S. Vergo, L. Groth-Pedersen, S. Eimer, A. Triller, C. Vannier, Regulation of gephyrin assembly and glycine receptor synaptic stability, J. Biol. Chem., 281 (2006) 30046-30056.
- [9] P. Legendre, E. Muller, C.I. Badiu, J. Meier, C. Vannier, A. Triller, Desensitization of homomeric alpha1 glycine receptor increases with receptor density, Mol. Pharmacol., 62 (2002) 817-827.
- [10] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell. Biol., 1 (2000) 31-39.
- [11] J.A. Allen, R.A. Halverson-Tamboli, M.M. Rasenick, Lipid raft microdomains and neurotransmitter signalling, Nat. Rev. Neurosci., 8 (2007) 128-140.
- [12] D. Choquet, Fast AMPAR trafficking for a high-frequency synaptic transmission, The European journal of neuroscience, 32 (2010) 250-260.
- [13] H.D. MacGillavry, J.M. Kerr, T.A. Blanpied, Lateral organization of the postsynaptic density, Mol. Cell. Neurosci., 48 (2011) 321-331.
- [14] J.C. Meier, C. Henneberger, I. Melnick, C. Racca, R.J. Harvey, U. Heinemann, V. Schmieden, R. Grantyn, RNA editing produces glycine receptor alpha3(P185L), resulting in high agonist potency, Nat Neurosci, 8 (2005) 736-744.
- [15] J. Kuhse, V. Schmieden, H. Betz, Identification and functional expression of a novel ligand binding subunit of the inhibitory glycine receptor, J. Biol. Chem., 265 (1990) 22317-22320.
- [16] Z. Nikolic, B. Laube, R.G. Weber, P. Lichter, P. Kioschis, A. Poustka, C. Mulhardt, C.M. Becker, The human glycine receptor subunit alpha3. Glra3 gene structure, chromosomal localization, and functional characterization of alternative transcripts, J. Biol. Chem., 273 (1998) 19708-19714.
- [17] S.A. Eichler, B. Forstera, B. Smolinsky, R. Juttner, T.N. Lehmann, M. Fahling, G. Schwarz, P. Legendre, J.C. Meier, Splice-specific roles of glycine receptor alpha3 in the hippocampus, Eur. J. Neurosci., 30 (2009) 1077-1091.
- [18] S.A. Eichler, S. Kirischuk, R. Juttner, P.K. Schafermeier, P. Legendre, T.N. Lehmann, T. Gloveli, R. Grantyn, J.C. Meier, Glycinergic tonic inhibition of hippocampal neurons with depolarizing GABAergic transmission elicits histopathological signs of temporal lobe epilepsy, J. Cell. Mol. Med., 12 (2008) 2848-2866.
- [19] P. Legendre, B. Forstera, R. Juttner, J.C. Meier, Glycine Receptors Caught between Genome and Proteome - Functional Implications of RNA Editing and Splicing, Front. Mol. Neurosci., 2 (2009) 23.

- [20] J.W. Lynch, Native glycine receptor subtypes and their physiological roles, Neuropharmacology, 56 (2009) 303-309.
- [21] D. Axelrod, D.E. Koppel, J. Schlessinger, E. Elson, W.W. Webb, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics, Biophys. J., 16 (1976) 1055-1069.
- [22] N. Smisdom, K. Braeckmans, H. Deschout, M. vandeVen, J.-M. Rigo, S.C. De Smedt, M. Ameloot, Fluorescence recovery after photobleaching on the confocal laser-scanning microscope: generalized model without restriction on the size of the photobleached disk, J. Biomed. Opt., 16 (2011) 046021-046028.
- [23] D. Alcor, G. Gouzer, A. Triller, Single-particle tracking methods for the study of membrane receptors dynamics, Eur. J. Neurosci., 30 (2009) 987-997.
- [24] C.M. Anderson, G.N. Georgiou, I.E. Morrison, G.V. Stevenson, R.J. Cherry, Tracking of cell surface receptors by fluorescence digital imaging microscopy using a charge-coupled device camera. Low-density lipoprotein and influenza virus receptor mobility at 4 degrees C, J. Cell. Sci., 101 (Pt 2) (1992) 415-425.
- [25] S. Wieser, G.J. Schutz, Tracking single molecules in the live cell plasma membrane-Do's and Don't's, Methods, 46 (2008) 131-140.
- [26] K. Peneva, G. Mihov, F. Nolde, S. Rocha, J. Hotta, K. Braeckmans, J. Hofkens, H. Uji-i, A. Herrmann, K. Mullen, Water-soluble monofunctional perylene and terrylene dyes: powerful labels for single-enzyme tracking, Angew. Chem. Int. Edit. Engl., 47 (2008) 3372-3375.
- [27] S. Rocha, J.A. Hutchison, K. Peneva, A. Herrmann, K. Mullen, M. Skjot, C.I. Jorgensen, A. Svendsen, F.C. De Schryver, J. Hofkens, H. Uji-i, Linking phospholipase mobility to activity by single-molecule wide-field microscopy, Chemphyschem, 10 (2009) 151-161.
- [28] Q. Tang, M. Edidin, Lowering the barriers to random walks on the cell surface, Biophys. J., 84 (2003) 400-407.
- [29] H. Qian, M.P. Sheetz, E.L. Elson, Single particle tracking. Analysis of diffusion and flow in two-dimensional systems, Biophys. J., 60 (1991) 910-921.
- [30] T.J. Feder, I. BrustMascher, J.P. Slattery, B. Baird, W.W. Webb, Constrained diffusion or immobile fraction on cell surfaces: A new interpretation, Biophys. J., 70 (1996) 2767-2773.
- [31] S.W. Paddock, Confocal laser scanning microscopy, BioTechniques, 27 (1999) 992-996, 998-1002, 1004.
- [32] W.B. Amos, J.G. White, How the confocal laser scanning microscope entered biological research, Biol. Cell., 95 (2003) 335-342.
- [33] D. Axelrod, Total internal reflection fluorescence microscopy in cell biology, Traffic, 2 (2001) 764-774.
- [34] N.L. Thompson, J.K. Pero, Total Internal Reflection Fluorescence Microscopy: Applications in Biophysics, in: M. Hof, R. Hutterer, V. Fidler (Eds.) Fluorescence Spectroscopy in Biology, vol. 3, Springer Berlin Heidelberg, 2005, pp. 79-103.
- [35] S.M. Simon, Partial internal reflections on total internal reflection fluorescent microscopy, Trends Cell. Biol., 19 (2009) 661-668.
- [36] N.O. Petersen, P.L. Hoddelius, P.W. Wiseman, O. Seger, K.E. Magnusson, Quantitation of membrane receptor distributions by image correlation spectroscopy: concept and application, Biophys. J., 65 (1993) 1135-1146.
- [37] P.W. Wiseman, N.O. Petersen, Image correlation spectroscopy. II. Optimization for ultrasensitive detection of preexisting platelet-derived growth factor-beta receptor oligomers on intact cells, Biophys. J., 76 (1999) 963-977.
- [38] C.M. Brown, R.B. Dalal, B. Hebert, M.A. Digman, A.R. Horwitz, E. Gratton, Raster image correlation spectroscopy (RICS) for measuring fast protein dynamics and concentrations with a commercial laser scanning confocal microscope, J. Microsc., 229 (2008) 78-91.
- [39] M.J. Rossow, J.M. Sasaki, M.A. Digman, E. Gratton, Raster image correlation spectroscopy in live cells, Nat. Protoc., 5 (2010) 1761-1774.
- [40] D.L. Kolin, S. Costantino, P.W. Wiseman, Sampling effects, noise, and photobleaching in temporal image correlation spectroscopy, Biophys. J., 90 (2006) 628-639.
- [41] J. Sankaran, M. Manna, L. Guo, R. Kraut, T. Wohland, Diffusion, transport, and cell membrane organization investigated by imaging fluorescence cross-correlation spectroscopy, Biophys. J., 97 (2009) 2630-2639.

- [42] E. Betzig, G.H. Patterson, R. Sougrat, O.W. Lindwasser, S. Olenych, J.S. Bonifacino, M.W. Davidson, J. Lippincott-Schwartz, H.F. Hess, Imaging intracellular fluorescent proteins at nanometer resolution, Science, 313 (2006) 1642-1645.
- [43] C. Flors, J. Hotta, H. Uji-i, P. Dedecker, R. Ando, H. Mizuno, A. Miyawaki, J. Hofkens, A stroboscopic approach for fast photoactivation-localization microscopy with Dronpa mutants, J. Am. Chem. Soc., 129 (2007) 13970-13977.
- [44] S.T. Hess, T.P. Girirajan, M.D. Mason, Ultra-high resolution imaging by fluorescence photoactivation localization microscopy, Biophys. J., 91 (2006) 4258-4272.
- [45] M.B. Roeffaers, G. De Cremer, J. Libeert, R. Ameloot, P. Dedecker, A.J. Bons, M. Buckins, J.A. Martens, B.F. Sels, D.E. De Vos, J. Hofkens, Super-resolution reactivity mapping of nanostructured catalyst particles, Angew. Chem. Int. Edit. Engl., 48 (2009) 9285-9289.
- [46] M.J. Rust, M. Bates, X.W. Zhuang, Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM), Nat. Methods, 3 (2006) 793-795.
- [47] M. Heilemann, S. van de Linde, A. Mukherjee, M. Sauer, Super-Resolution Imaging with Small Organic Fluorophores, Angew. Chem. Int. Edit. Engl., 48 (2009) 6903-6908.
- [48] M. Heilemann, S. van de Linde, M. Schuttpelz, R. Kasper, B. Seefeldt, A. Mukherjee, P. Tinnefeld, M. Sauer, Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes, Angew. Chem. Int. Edit. Engl., 47 (2008) 6172-6176.
- [49] S. van de Linde, A. Loschberger, T. Klein, M. Heidbreder, S. Wolter, M. Heilemann, M. Sauer, Direct stochastic optical reconstruction microscopy with standard fluorescent probes, Nat. Protoc., 6 (2011) 991-1009.
- [50] S. van de Linde, I. Krstic, T. Prisner, S. Doose, M. Heilemann, M. Sauer, Photoinduced formation of reversible dye radicals and their impact on super-resolution imaging, Photoch. Photobio. Sci., 10 (2011) 499-506.
- [51] K.I. Mortensen, L.S. Churchman, J.A. Spudich, H. Flyvbjerg, Optimized localization analysis for single-molecule tracking and super-resolution microscopy, Nat. Methods, 7 (2010) 377-381.
- [52] R.E. Thompson, D.R. Larson, W.W. Webb, Precise nanometer localization analysis for individual fluorescent probes, Biophys. J., 82 (2002) 2775-2783.
- [53] M. Ehrlich, W. Boll, A. van Oijen, R. Hariharan, K. Chandran, M.L. Nibert, T. Kirchhausen, Endocytosis by random initiation and stabilization of clathrin-coated pits, Cell, 118 (2004) 591-605.
- [54] B.D. Ripley, 2nd-Order Analysis of Stationary Point Processes, J. Appl. Probab., 13 (1976) 255-266.
- [55] B.D. Ripley, Modeling Spatial Patterns, J Roy Stat Soc B Met, 39 (1977) 172-212.
- [56] R.D. Phair, S.A. Gorski, T. Misteli, Measurement of dynamic protein binding to chromatin in vivo, using photobleaching microscopy, Methods. Enzymol., 375 (2004) 393-414.
- [57] R.D. Phair, T. Misteli, High mobility of proteins in the mammalian cell nucleus, Nature, 404 (2000) 604-609.
- [58] K. Braeckmans, L. Peeters, N.N. Sanders, S.C. De Smedt, J. Demeester, Three-dimensional fluorescence recovery after photobleaching with the confocal scanning laser microscope, Biophys. J., 85 (2003) 2240-2252.
- [59] E. Gielen, N. Smisdom, M. vandeVen, B. De Clercq, E. Gratton, M. Digman, J.M. Rigo, J. Hofkens, Y. Engelborghs, M. Ameloot, Measuring Diffusion of Lipid-like Probes in Artificial and Natural Membranes by Raster Image Correlation Spectroscopy (RICS): Use of a Commercial Laser-Scanning Microscope with Analog Detection, Langmuir, 25 (2009) 5209-5218.
- [60] J. Sankaran, X. Shi, L.Y. Ho, E.H. Stelzer, T. Wohland, ImFCS: a software for imaging FCS data analysis and visualization, Opt. Express, 18 (2010) 25468-25481.
- [61] T.Y. Kim, H. Uji-i, M. Moller, B. Muls, J. Hofkens, U. Alexiev, Monitoring the interaction of a single G-protein key binding site with rhodopsin disk membranes upon light activation, Biochemistry, 48 (2009) 3801-3803.
- [62] M.J. Saxton, Single-particle tracking: the distribution of diffusion coefficients, Biophys. J., 72 (1997) 1744-1753.

- [63] P.R. Smith, I.E. Morrison, K.M. Wilson, N. Fernandez, R.J. Cherry, Anomalous diffusion of major histocompatibility complex class I molecules on HeLa cells determined by single particle tracking, Biophys. J., 76 (1999) 3331-3344.
- [64] N. Unwin, Refined structure of the nicotinic acetylcholine receptor at 4A resolution, J. Mol. Biol., 346 (2005) 967-989.
- [65] J. Kistler, R.M. Stroud, M.W. Klymkowsky, R.A. Lalancette, R.H. Fairclough, Structure and function of an acetylcholine receptor, Biophys. J., 37 (1982) 371-383.
- [66] P. Annibale, S. Vanni, M. Scarselli, U. Rothlisberger, A. Radenovic, Identification of clustering artifacts in photoactivated localization microscopy, Nat. Methods, 8 (2011) 527-528.
- [67] M.A. Kiskowski, J.F. Hancock, A.K. Kenworthy, On the use of Ripley's K-function and its derivatives to analyze domain size, Biophys. J., 97 (2009) 1095-1103.
- [68] J.F. Nagle, Long tail kinetics in biophysics?, Biophys. J., 63 (1992) 366-370.
- [69] D.E. Wolf, Designing, building, and using a fluorescence recovery after photobleaching instrument, Methods. Cell. Biol., 30 (1989) 271-306.
- [70] M.A. Digman, C.M. Brown, P. Sengupta, P.W. Wiseman, A.R. Horwitz, E. Gratton, Measuring fast dynamics in solutions and cells with a laser scanning microscope, Biophys. J., 89 (2005) 1317-1327.
- [71] E. Gielen, N. Smisdom, B. De Clercq, M. Vandeven, R. Gijsbers, Z. Debyser, J.M. Rigo, J. Hofkens, Y. Engelborghs, M. Ameloot, Diffusion of myelin oligodendrocyte glycoprotein in living OLN-93 cells investigated by raster-scanning image correlation spectroscopy (RICS), J. Fluoresc., 18 (2008) 813-819.
- [72] M.J. Saxton, Single-particle tracking: effects of corrals, Biophys. J., 69 (1995) 389-398.
- [73] R. Simson, E.D. Sheets, K. Jacobson, Detection of temporary lateral confinement of membrane proteins using single-particle tracking analysis, Biophys. J., 69 (1995) 989-993.
- [74] K.M. Wilson, I.E.G. Morrison, P.R. Smith, N. Fernandez, R.J. Cherry, Single particle tracking of cell-surface HLA-DR molecules using R-phycoerythrin labeled monoclonal antibodies and fluorescence digital imaging, J. Cell. Sci., 109 (1996) 2101-2109.
- [75] P. Thomas, T.G. Smart, HEK293 cell line: a vehicle for the expression of recombinant proteins, J. Pharmacol. Toxicol. Methods., 51 (2005) 187-200.
- [76] A. Kusumi, C. Nakada, K. Ritchie, K. Murase, K. Suzuki, H. Murakoshi, R.S. Kasai, J. Kondo, T. Fujiwara, 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., 34 (2005) 351-378.
- [77] Y. Gambin, R. Lopez-Esparza, M. Reffay, E. Sierecki, N.S. Gov, M. Genest, R.S. Hodges, W. Urbach, Lateral mobility of proteins in liquid membranes revisited, Proc. Natl. Acad. Sci. U. S. A., 103 (2006) 2098-2102.
- [78] M. Dahan, S. Levi, C. Luccardini, P. Rostaing, B. Riveau, A. Triller, Diffusion dynamics of glycine receptors revealed by single-quantum dot tracking, Science, 302 (2003) 442-445.
- [79] M.V. Ehrensperger, C. Hanus, C. Vannier, A. Triller, M. Dahan, Multiple association states between glycine receptors and gephyrin identified by SPT analysis, Biophys. J., 92 (2007) 3706-3718.
- [80] C. Charrier, M.V. Ehrensperger, M. Dahan, S. Levi, A. Triller, Cytoskeleton regulation of glycine receptor number at synapses and diffusion in the plasma membrane, J. Neurosci., 26 (2006) 8502-8511.
- [81] M.D. Ehlers, M. Heine, L. Groc, M.C. Lee, D. Choquet, Diffusional trapping of GluR1 AMPA receptors by input-specific synaptic activity, Neuron, 54 (2007) 447-460.
- [82] H.G. Breitinger, C.M. Becker, The inhibitory glycine receptor-simple views of a complicated channel, Chembiochem : a European journal of chemical biology, 3 (2002) 1042-1052.
- [83] P. Legendre, The glycinergic inhibitory synapse, Cellular and molecular life sciences : CMLS, 58 (2001) 760-793.
- [84] H. Mizuno, M. Abe, P. Dedecker, A. Makino, S. Rocha, Y. Ohno-Iwashita, J. Hofkens, T. Kobayashi, A. Miyawaki, Fluorescent probes for superresolution imaging of lipid domains on the plasma membrane, Chem. Sci., 2 (2011) 1548-1553.

- [85] K. Burger, G. Gimpl, F. Fahrenholz, Regulation of receptor function by cholesterol, Cellular and molecular life sciences : CMLS, 57 (2000) 1577-1592.
- [86] R. Willmann, S. Pun, L. Stallmach, G. Sadasivam, A.F. Santos, P. Caroni, C. Fuhrer, Cholesterol and lipid microdomains stabilize the postsynapse at the neuromuscular junction, The EMBO journal, 25 (2006) 4050-4060.
- [87] H.G. Breitinger, C. Villmann, N. Melzer, J. Rennert, U. Breitinger, S. Schwarzinger, C.M. Becker, Novel regulatory site within the TM3-4 loop of human recombinant alpha3 glycine receptors determines channel gating and domain structure, The Journal of biological chemistry, 284 (2009) 28624-28633.





















# Ensemble and single particle fluorimetric techniques in concerted action to study the diffusion and aggregation of the glycine receptor $\alpha 3$ isoforms in the cell plasma membrane

Kristof Notelaers<sup>\*a,b</sup>, Nick Smisdom<sup>\*a</sup>, Susana Rocha<sup>b</sup>, Daniel Janssen<sup>a</sup>, Jochen C. Meier<sup>c</sup>, Jean-Michel Rigo<sup>a</sup>, Johan Hofkens<sup>b</sup>, Marcel Ameloot<sup>a</sup>

- a Biomedical Research Institute, Hasselt University and School of Life Sciences, transnational University Limburg, Agoralaan gebouw C, 3590 Diepenbeek, Belgium
- b Laboratory for Photochemistry and Spectroscopy, Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200F, B-3001 Heverlee, Belgium
- c RNA Editing and Hyperexcitability Disorders Group, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13092 Berlin, Germany
- \* authors contributed equally

Corresponding author: Marcel Ameloot Agoralaan gebouw C 3590 Diepenbeek Belgium telephone number: +32 11 26 92 33 fax number: +32 11 26 92 99 e-mail: marcel.ameloot@uhasselt.be

# Supplementary material 1: FRAP analysis: a new, robust method to select a reference region

Analysis of a FRAP experiment carried out on a confocal laser scanning microscope consists of two steps. In the first step, a normalized recovery curve is computed from the acquired time series of images. This is followed in the second step by a fit of this obtained recovery curve to an appropriate model yielding the parameters of interest like the diffusion coefficient D and immobile fraction M. The latter step has received a considerable amount of attention resulting in more accurate theoretical models.[1,2] However, these efforts would be in vain when the recovery curve is not calculated with the same highest punctuality available.

To this end, Smisdom et al. introduced a straightforward method to estimate the experimental error of an individual recovery curve [2] without the necessity of reproducing the experiment under exactly identical conditions, which is difficult when working with biological cells. The resulting errors can afterwards being included in the fit of the experimental data to the theoretical model. The error estimation approach brings into account all errors originating from the experimental limitations of the detection system as well as their propagation throughout further calculations. These further calculations encompass all corrections based on the use of a representative reference region, as will be discussed later on. The selection of this reference region seems straightforward and little attention is devoted to it, but it can actually be a major factor distorting the final data.

For an ideal system the reference region would not be required. In that case it would be sufficient to normalize the raw recovery curve  $F_r(t)$  to its mean pre-bleach intensity  $F_{r,o}$ , a procedure known as single normalization.[3] However, in real experiments artifacts like photobleaching during data acquisition and background contribution can hardly ever be excluded. To cope with these experimental limitations, a double normalization is suggested.[4] In this approach, two extra regions are extracted from the time series of images: one reference region *R* and one background region *B*.

The reference region R is used to correct for instrumental fluctuations and bleaching during acquisition. This region should fulfill two requirements. First, it should hold a region for which a constant intensity can be inferred. In this way, any deviation from its initial intensity can be attributed to experimental limitations. And secondly, R should be representative for the bleach region, i.e. it should experience identical conditions as the bleach region, except for the FRAP bleaching. Furthermore, also the sample dynamics in R have to be identical to those in the bleach region.

The background region B has to comprise a region devoid of any intensity contribution derived from the fluorescent species. The remaining intensity can be of instrumental (e.g. digital intensity offset, excitation light scattering) or biological origin (autofluorescence).

Using the average intensities derived from the three regions, the double normalization can be performed as:[4]

$$\frac{F(t)}{F_o} = \frac{F_r(t) - B(t)}{F_o[R(t) - B(t)]}$$
(1.1)

where the corrected mean pre-bleach intensity  $F_o$  is calculated as:

$$F_o = \left\langle \frac{F_{r,o}(t) - B(t)}{R(t) - B(t)} \right\rangle \quad , \quad t < 0 \tag{1.2}$$

The angular brackets denote a time averaging of the pre-bleach intensity. t = 0 at the onset of the photobleaching.

Although the extraction and calculation of the recovery curve seems to be straightforward, an extra difficulty arises when studying the diffusion or proteins in the plasma membrane of live cells. To correct for instrumental fluctuations and membrane undulations, the reference region needs to be defined. The most prevalent approach is to select a region sufficiently far from the bleach region (*Figure 1 a*). Due to the spatial heterogeneity of cells, the intensity of each part of the cell membrane appears not to have an identical time course. This becomes evident when comparing the results from the recovery curves obtained with distinct reference regions. These results can easily differ one order of magnitude.

In an attempt to rationalize the selection of the reference region, a method was created that generates a mask of the cell and that excludes the bleach region and a sufficient perimeter around it (*Figure 1 b*). Although this approach brings a lot more cell membrane into account, the resulting recovery curve often suffers from severe fluctuations. This can probably be attributed to the heterogeneity of the cell membrane.

Finally, a third method was developed, which is partially based on the method to select the reference region when studying nuclear proteins.[3] For nuclear proteins, typically the complete nucleus is selected as reference region. Analogously, the complete cell could be selected. However, based on the previous method (*Figure 1 b*), it is known that this approach is not successful. A solution was found by modifying Phair's approach by selecting a reference region that comprises the bleach region together with its perimeter (*Figure 1 c*). For a sufficiently large perimeter, the fluorescence intensity after bleaching can be assumed to be constant. Sufficiently large means that no bleached fluorophore can leave the reference region during the time span of the experiment and depends on experimental parameters like the diffusion coefficient. For data points before and right after bleaching, this reference region is modified by selecting only a rim of the perimeter. The resulting approach allows to extract the recovery curve independent from the user's impression. Furthermore, since only the region in the vicinity of the bleach region is used, the reference region can be expected to better mimic the bleach region.



Figure 1: Schematic illustration of the selection of the reference region

Selection of the reference region by manually selecting the region at sufficient distance from the bleach region. b) Using a mask to select the reference region, also sufficiently far from the bleach region. c) selecting a region that comprises the bleach region and its perimeter in which the fluorescence can be assumed to be constant after bleaching occurred.

#### Supplementary material 2: Expression and functionality check of glycine receptors.

# 2.1. Western blot

The heterologous expression of GlyR  $\alpha$ 3K and  $\alpha$ 3L was compared with the expression of wild type GlyR by means of western blot analysis. For each condition, 3 million cells were plated in small culture flasks (25 cm<sup>2</sup>). Transfection of the cells occurred as described above. After 24 hours of transfection, cells were rinsed with cold (4°C) phosphate buffered saline (PBS) and trypsinated. The cell suspensions were centrifuged and rinsed again with PBS. After a second centrifugation step, cells were lysed by addition of RIPA buffer (Sigma-Aldrich, Bornem, Belgium). Protein concentrations were determined using the BCA<sup>TM</sup> Protein Assay kit (Pierce, Rockford, IL, USA). For each condition, 10, 25 and 50 µg of total protein content was subjected to precast 8% Novex® Tris-Glycine gel electrophoresis using an XCell SureLock<sup>TM</sup> Mini-Cell. The proteins were subsequently transferred onto a nitrocellulose transfer membrane via the iBlot<sup>TM</sup> Dry blotting System (Invitrogen/Molecular Probes). After blocking with 2% non-fat milk in PBS (overnight at 4°C), the membranes were incubated with mAb4a (1:500, Pfeiffer et al 1984, Synaptic Systems, Goettingen, Germany) followed by a peroxidase-conjugated secondary antibody (Rabbit anti-mouse 1:1000, Dakocytomation, Heverlee, Belgium) and finally the bands were visualized using a 3,3'-Diaminobenzidine (DAB, Sigma-Aldrich).



#### Figure 2: Expression of the GlyR α3 isoforms in HEK 293 cells

The expression of both GlyR  $\alpha$ 3K and  $\alpha$ 3L subunits after transfection was confirmed using western blot. The band of both receptors is located between 40 and 50 kDa, as expected. Furthermore, due to the extra amino acids, the band of GlyR  $\alpha$ 3L is located at higher molecular weights as compared to GlyR  $\alpha$ 3K. The antibody mAb4a was used to recognize all GlyRs present in the cell lysates. No significant expression was detected of endogenous GlyRs by wild-type HEK 293 cells. (The contrast of a small stripe of the protein standard lane was digitally enhanced to improve the visualization of the protein standard.)

# 2.2. Electrophysiology

Cells seeded in 35 mm dishes, were transfected as described above and whole-cell patch-clamp recordings were performed (EPC 10, HEKA Elektronik, Lambrecht, Pfalz, Germany). The standard extracellular solution for HEK cells (HES) had a composition of (in mM) 150 NaCl, 5.4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, and 10 glucose (Sigma-Aldrich). The intracellular solution (HIS) contained (in mM): 120 CsCl, 2 Na<sub>2</sub>ATP, 2 MgATP, 10 EGTA, and 10 HEPES. To measure the dose-response to glycine, different glycine concentrations (1  $\mu$ M, 10  $\mu$ M, 30  $\mu$ M, 100  $\mu$ M, 300  $\mu$ M and 1 mM) in HES were bath-applied onto the cell surface during 10 s at a holding potential of -60 mV. The average normalized maximum current as a function of concentration was fit to the *Hill* equation to yield the EC<sub>50</sub> of both splice variants (GraphPad Prism, La Jolla, CA, USA). The desensitization of the glycine response at room temperature.





Current traces of responses were induced by different concentrations of glycine for GlyR  $\alpha$ 3K (a) and GlyR  $\alpha$ 3L (b). The dose-response curve of both receptors is shown in (c). Mean values ± SEM are plotted. A nonlinear fit according to the Hill equation revealed an EC50 of 47 ± 10 µM and Hill slope 1.5 (n=6) for the K splice variant and an EC50 52 ± 24 µM and Hill slope 1.2 (n=6) for the L splice variant. The time constant of the desensitization as a function of the glycine concentration of the two isoforms is plotted in (d). A dosedependent desensitization was present. Moreover, GlyR  $\alpha$ 3L desensitized more slowly than GlyR  $\alpha$ 3K. (\*: P < 0.05)

#### 2.3. Functional GlyR α3 expression in HEK 293 cells.

Expression of both heterologous HA-GlyR  $\alpha 3$  isoforms was confirmed by western blot analysis (*Figure 2*). The measured molecular weight of the receptors between 40 and 50 kDa is as expected, with the band of GlyR  $\alpha 3L$  slightly elevated compared to that of GlyR  $\alpha 3K$  due to the presence of extra amino acids[5,6]. No endogeneous GlyR expression is found by the mAb4a antibody, which recognizes an endogenous epitope common to all glycine receptors  $\alpha$ -subunits [7-9].

The functionality of the expressed receptors was evaluated by whole-cell patch-clamp. Current traces exhibited a dose-dependent response to glycine for both subunit isoforms, with an EC50 of 47 ± 10  $\mu$ M (n=6) for GlyR  $\alpha$ 3K and 52 ± 24  $\mu$ M (n=6) for GlyR  $\alpha$ 3L (3 *a,b,c*). The time constant ( $\tau$ ) of the receptor desensitization for the HA-GlyR  $\alpha$ 3L ( $\tau$  = 7.86 ± 0.09 *s* at 100  $\mu$ M and 5.3 ± 0.7 *s* at 300  $\mu$ M glycine) was significantly longer as compared to the HA-GlyR  $\alpha$ 3K ( $\tau$  = 2.3 ± 0.9 *s* at 100  $\mu$ M and 1.8 ± 0.4 *s* at 300  $\mu$ M glycine) (3 d). This is in agreement with published results [10].

#### Supplementary material 3: Effect of photobleaching on TICS measurements

Temporal image correlation spectroscopy (TICS) is considered as the imaging analogue of Fluorescence correlation spectroscopy (FCS) and is therefore also called imFCS.[11] A schematic comparison between both techniques is depicted in *Figure 4*.





TICS can be considered as a multiplex FCS experiment in which the single observation volume (a) is substituted by an array of up to 105 observation volumes (b, pictorial representation). The fluorescence intensity time series of FCS (c) is consequently replaced by a time series of images (d). Each pixel location in this time series can yield an ACF (f) which can all be averaged to obtain a single ACF as in FCS (e).

Since TICS is closely related to FCS, it has some advantages but also some limitations in common. One of these limitations is its sensitivity to photobleaching. In the presence of photobleaching, one can expect to find an apparently higher *D*. In addition to leaving the observation area by diffusion, an extra pathway to disappear from this area exists when photobleaching occurs. Furthermore, the decreasing number of fluorescent particles will lead to an increase of the temporal autocorrelation function (TACF) amplitude.[12] When photobleaching is minimal, it is often ignored.

To cope with the artifacts induced by photobleaching, the problem can be divided into two aspects: correct calculation of the TACF on one hand and its correct interpretation on the other.[12] For FCS, where a time average is used, the calculation of the ACF is incorrect. The time average will no longer be representative for the ensemble average due to the decreasing fluorescence intensity over time. Two

frequently applied solutions are piecewise analysis of the fluorescence intensity time series or a correction of the fluorescence intensity over time prior to ACF calculation.[12] Although both methods allow for the correct calculation of the autocorrelation function (ACF), they do not correct for the decaying concentration of the fluorescent species. TICS can be applied using a similar time average approach, yielding the same limitations. However, TICS can also be applied using spatial averages, which makes it more feasible to tackle the problem. When each position in the sample experiences identical bleaching, this approach will remain valid. As a result, only the correction of the TACF should be considered.

The ACF in the presence of photobleaching,  $g_{pb}(\tau)$ , can be corrected for a decaying average number in the observation area (*N*) by:[12]

$$g(\tau) = g_{pb}(\tau) \langle [\langle b(t) \rangle \langle b(t+\tau) \rangle]^{-1/2} \rangle^{-1}$$
(4.1)

where b(t) is the time course of N during the measurement normalized to the initial occupation number  $N_0$ . For photobleaching, b(t) often results in a mono- or bi-exponential decay. It has been shown that a correction similar to Eq. (4.1) is also possible for TICS.[13] However, the final expression in [13] does not bring  $\tau$  into account. Based on Eq. (4.1) it is reasonable to assume that this might lead to errors, especially when  $\tau \ll T$ . Therefore, the problem is reconsidered.

The average, spatial image intensity can be expressed in terms of the average occupation number and the brightness of the fluorescent species:

$$\langle i(x, y, t) \rangle_{XY} = \varepsilon \langle N(x, y, t) \rangle_{XY}$$
(4.2)

where *N* is now assumed to be a function of space and time. To bring into account the fluctuation in each pixel, the pixel intensity i(x, y, t) can be written as:

$$i(x, y, t) = \varepsilon \langle N(x, y, t) \rangle_{XY} + \mathcal{G}(x, y, t) \varepsilon \sqrt{\langle N(x, y, t) \rangle_{XY}}$$
(4.3)

where  $\vartheta(x, y, t)$  represents the normalized thermodynamical fluctuation[12] related to  $\langle N(x, y, t) \rangle_{xy} = 1$ , i.e.  $\langle \vartheta(x, y, t)^2 \rangle_{xy} = 1$ . The fluctuations around the mean can be calculated as

$$\delta i(x, y, t) = i(x, y, t) - \langle i(x, y, t) \rangle_{XY}$$
(4.4)

Substitution of Eq. (4.2) and Eq. (4.3) into Eq. (4.4) yields the intensity fluctuations around the average as a function of N:

$$\delta i(x, y, t) = \vartheta(x, y, t) \varepsilon \sqrt{\langle N(x, y, t) \rangle_{XY}}$$
(4.5)

Based on this definition, the TACF can be calculated as:

$$g(0,0,\tau) = \left\langle \frac{\left\langle \vartheta(x,y,t)\varepsilon\sqrt{\langle N(x,y,t)\rangle_{XY}}\,\vartheta(x,y,t+\tau)\varepsilon\sqrt{\langle N(x,y,t+\tau)\rangle_{XY}}\,\right\rangle_{XY}}{\varepsilon\langle N(x,y,t)\rangle_{XY}\,\varepsilon\langle N(x,y,t)\rangle_{XY}}\,\right\rangle_{T}$$

$$= h(0,0,\tau) \left\langle \frac{\sqrt{\langle N(x,y,t)\rangle_{XY}}\,\sqrt{\langle N(x,y,t+\tau)\rangle_{XY}}}{\langle N(x,y,t)\rangle_{XY}\,\langle N(x,y,t)\rangle_{XY}}\,\right\rangle_{T}$$

$$= h(0,0,\tau) \left\langle \frac{1}{\langle N(x,y,t)\rangle_{XY}\,\langle N(x,y,t)\rangle_{XY}}\,\right\rangle_{T}$$

$$(4.6)$$

where  $h(0,0,\tau) = \langle \langle \vartheta(x,y,t)\vartheta(x,y,t+\tau) \rangle_{XY} \rangle_T$ .

Since it is assumed that all pixels experience an identical bleaching,  $\langle N(x, y, t) \rangle_{xy}$  can be expressed as the product of the initial spatial average occupation number  $N_o$  with a bleach function b(t), with b(0)=1:

$$\langle N(x, y, t) \rangle_{XY} = N_o b(t)$$
 (4.7)

Eq. (4.6) can subsequently be expressed in terms of  $N_o$  and b(t):

$$g_{pb}(0,0,\tau) = \frac{h(0,0,\tau)}{N_o} \langle [b(t)b(t+\tau)]^{-1/2} \rangle_T$$

$$= g(0,0,\tau) \langle [b(t)b(t+\tau)]^{-1/2} \rangle_T$$
(4.8)

In comparison with Eq. (4.1), not the product of the time averages but the time average of the product is used. The TACF corrected for photobleaching can thus be obtained as:

$$g(0,0,\tau) = \frac{g_{pb}(0,0,\tau)}{\left< \left[ b(t)b(t+\tau) \right]^{-\frac{1}{2}} \right>_{T}}$$
(4.9)

Although b(t) is assumed to be a decaying function in case of photobleaching, it can be any function representing a general trend in the observed occupation number during the measurement.

The effect of a mono-exponential bleaching with various bleaching rates was investigated, highlighting the robustness of the correction.[13] Unfortunately, the results were limited to specific experimental conditions. Therefore, the limitations of the correction are revised here.

The correction relies essentially on the fluctuations of the remaining fluorescent particles. It is reasonable to assume that the correction fails when all fluorescence is bleached. Furthermore, deviations will start to arise when the fractional intensity drops below 1/N. As a result, higher densities will allow for a better bleach correction.

The effect of the bleach rate was studied by us by using computer simulations. Stacks of 4000 images were simulated with a density of 10 *particles/µm*<sup>2</sup>, 512×512 resolution,  $\omega_0 = 400 \text{ nm}$  and d = 80 nm. D was set to 0.1  $\mu m^2/s$  and the interval between frames was kept constant at  $\frac{1}{4}\tau_D = 0.1 \text{ s}$ . The effect of the finite pixel size was ignored. The bleaching was applied as a mono-exponential:

$$b(t) = e^{-kt} \tag{4.10}$$

where *k* is the bleaching decay constant with reciprocal time units. *k* ranged from 0 to 1  $s^{-1}$ . Each stack of images was analyzed repetitively while varying the number of frames included in the analysis. The analysis comprised calculation of the TACF, fit of the fluorescence decrease with a mono-exponential including a constant offset, correction of the TACF according to Eq. (4.9) and fit of both the original and corrected TACF [*Figure 5*].



Figure 5: Exemplar curves for simulated fluorescence decrease and TACF

a) Time course of fluorescence for eight different stacks of simulated images. The bleaching rate k is expressed as the reciprocal of for generality. The unweighted residuals between the fit of the decay and the simulated values are shown in the top left panel. b) The TACF of the first 100 frames associated with four different bleaching rates. The difference in amplitude is obvious. c) identical TACFs as in b), but corrected using the method described above [Eq (4.9)].

The normalized deviation of the obtained results in comparison to the simulation parameters where subsequently plotted as a function of bleaching rate and the number of frames included in the TACF calculation [*Figure 6*]. It is obvious that an increasing number of frames included in the analysis has a negative effect on the recovery of both the occupation number and  $\tau_D$ . This is in contrast with the situation in absence of photobleaching, where the uncertainty on the recovered parameter values is reduced by increasing the number of frames included in the analysis.

As expected, *N* is underestimated for high *k*. The bleach correction extends the range of bleach rates that can still be tolerated by about one decade. For  $\tau_D$ , in contrast, no obvious improvement is detected by applying the bleach correction. The simulations show an increase of  $\tau_D$  with increasing *k*.





The TACFs of the simulated data sets were fit and normalized deviations were plotted as a function of k. The analyses also included six different numbers of frames, each depicted in a different color. (a) For uncorrected TACFs, the occupation number is underestimated for large k, which is further aggravated when more frames are included in the analysis. (b) The bleach correction reduces the deviation and extends the bleaching range that still allows for a correct analysis. (c) and (d) show the result for  $\tau_D$  as a function of k obtained from respectively uncorrected and corrected TACFs. Also here, the effect of the number of included frames is obvious. The correction, however, does apparently not result in a reduction of the error for large k.

The number of frames included in the analysis determines the maximum k that can be corrected for. Since this number depends on experimental settings, the simulation are further generalized by plotting the result as a function of the amount of fluorescence that is lost during the acquisition [*Figure 7*]. These results demonstrate that the underestimation of N is independent of k and depends solely on the loss of fluorescence during the acquisition. Again, the bleach correction reduces the normalized error by one decade. Also for  $\tau_D$ , an increase in the loss of fluorescence aggravates the error, which again cannot be corrected by the bleach correction method.

Based on these results, it is obvious that the loss of fluorescence during the acquisition has to be minimized. For high bleaching rates, a solution can be found by limiting the number of frames included in the analysis. A loss of fluorescence less than 50% yields, after correction, parameter estimates with less than 10% of normalized errors for high *k*. Unfortunately, this approach has a tradeoff. By reducing the number of frames the number of observed  $\tau_D$  will also decrease, leading again to an increase of the uncertainty.



Figure 7: Deviation on the retrieved parameters as a function of lost fluorescence

The normalized deviations were plotted as a function of k. Instead of considering the number of frames included in the analysis, the fluorescence intensity was used as a criterion to determine this number. The black curve indicates the limit of the analysis, which is 4000 frames. (a) For uncorrected TACFs, the underestimation of the occupation number increases with increasing loss of fluorescence. This is constant for all k. (b) The bleach correction reduces the normalized deviation by one decade. (c) and (d) show the result for  $\tau_D$  as a function of k obtained from respectively uncorrected and correction, however, does apparently not result in a reduction of the error for large k.

# References

- [1] K. Braeckmans, L. Peeters, N.N. Sanders, S.C. De Smedt, J. Demeester, Three-dimensional fluorescence recovery after photobleaching with the confocal scanning laser microscope, Biophys. J., 85 (2003) 2240-2252.
- [2] N. Smisdom, K. Braeckmans, H. Deschout, M. vandeVen, J.-M. Rigo, S.C. De Smedt, M. Ameloot, Fluorescence recovery after photobleaching on the confocal laser-scanning microscope: generalized model without restriction on the size of the photobleached disk, J. Biomed. Opt., 16 (2011) 046021-046028.
- [3] R.D. Phair, S.A. Gorski, T. Misteli, Measurement of dynamic protein binding to chromatin in vivo, using photobleaching microscopy, Methods. Enzymol., 375 (2004) 393-414.
- [4] R.D. Phair, T. Misteli, High mobility of proteins in the mammalian cell nucleus, Nature, 404 (2000) 604-609.
- [5] G. Grenningloh, I. Pribilla, P. Prior, G. Multhaup, K. Beyreuther, O. Taleb, H. Betz, Cloning and expression of the 58 kd beta subunit of the inhibitory glycine receptor, Neuron, 4 (1990) 963-970.
- [6] G. Grenningloh, A. Rienitz, B. Schmitt, C. Methfessel, M. Zensen, K. Beyreuther, E.D. Gundelfinger, H. Betz, The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors, Nature, 328 (1987) 215-220.
- [7] U. Grunert, H. Wassle, Immunocytochemical Localization of Glycine Receptors in the Mammalian Retina, Journal of Comparative Neurology, 335 (1993) 523-537.
- [8] F. Pfeiffer, R. Simler, G. Grenningloh, H. Betz, Monoclonal-Antibodies and Peptide-Mapping Reveal Structural Similarities between the Subunits of the Glycine Receptor of Rat Spinal-Cord, P. Natl. Acad. Sci.-Biol., 81 (1984) 7224-7227.
- [9] S. Schroder, W. Hoch, C.M. Becker, G. Grenningloh, H. Betz, Mapping of Antigenic Epitopes on the Alpha-1 Subunit of the Inhibitory Glycine Receptor, Biochemistry, 30 (1991) 42-47.
- [10] Z. Nikolic, B. Laube, R.G. Weber, P. Lichter, P. Kioschis, A. Poustka, C. Mulhardt, C.M. Becker, The human glycine receptor subunit alpha3. Glra3 gene structure, chromosomal localization, and functional characterization of alternative transcripts, J. Biol. Chem., 273 (1998) 19708-19714.
- [11] J. Sankaran, X. Shi, L.Y. Ho, E.H. Stelzer, T. Wohland, ImFCS: a software for imaging FCS data analysis and visualization, Opt. Express, 18 (2010) 25468-25481.
- [12] A. Delon, Y. Usson, J. Derouard, T. Biben, C. Souchier, Photobleaching, mobility, and compartmentalisation: inferences in fluorescence correlation spectroscopy, J. Fluoresc., 14 (2004) 255-267.
- [13] D.L. Kolin, S. Costantino, P.W. Wiseman, Sampling effects, noise, and photobleaching in temporal image correlation spectroscopy, Biophys. J., 90 (2006) 628-639.