Monitoring intracellular calcium in response to GPCR activation using thin-film silicon photodiodes with integrated fluorescence filters

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Abstract

G-protein coupled receptor (GPCRs) drug discovery is a thriving strategy in the pharmaceutical industry. The standard approach uses living cells to test millions of compounds in a high-throughput format. Typically, changes in the intracellular levels of key elements in the signaling cascade are monitored using fluorescence or luminescence read-out systems, which require external equipment for signal acquisition. In this work, thin-film amorphous silicon photodiodes with an integrated fluorescence filter were developed to capture the intracellular calcium dynamics in response to the activation of the endogenous muscarinic M1 GPCR of HEK 293T cells. Using the new device it was possible to characterize the potency of carbachol (EC50=10.5 μM) and pirenzepine (IC50=4.2 μM), with the same accuracy as standard microscopic optical systems. The smaller footprint provided by the detection system makes it an ideal candidate for the future integration in microfluidic devices for drug discovery.

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1. Introduction

The use of cells in biosensing and assaying often relies on the ability of their natural chemical receptors to respond to small variations in the quantities of target molecules in the surrounding environment. In eukaryotic cells, G-protein coupled receptors (GPCRs) comprise one of the largest classes of such receptors (Lagerstrom and Schioth, 2008; Lefkowitz, 2007). Ligand binding to GPCRs induces conformational changes on the receptor which leads to the activation of a transducer, a heterotrimeric G protein. The latter, in turn, activates an effector enzyme with the subsequent generation of second messenger molecules such as inositol triphosphate (IP3), calcium (Ca2+)-cyclic adenosine monophosphate (cAMP), that alter cell activity (Eglen et al., 2007). GPCRs are thus extremely important targets for drug design, accounting for more than 30% of the pharmaceutical molecules currently available (Hopkins and Groom, 2002). Moreover, specific ligands have not yet been identified for more than 100 GPCRs (orphan GPCRs) (Kenakin, 2009; Schlyer and Horuk, 2006). Given this context, the ability to screen libraries of compounds for potential GPCR-acting molecules is crucial.

While the use of in silico approaches based on receptor structure models or computational approaches for rational drug design are emerging (Lagerstrom and Schioth, 2008; Shoichet and Kobilka, 2012), the current GPCR drug discovery programs are supported by the screening of millions of small molecule libraries either in high throughput (HTS) or high content (HCS) screening formats (Sewing and Cawkill, 2007). Historically, radioligand binding assays were the method of choice for the identification of compounds that bind to GPCR. Albeit sensitive, the relatively high costs, the limited compatibility with high throughput formats (Sewing and Cawkill, 2007) and hazardous protocols have prompted the development of non-radioactive alternatives (Zhang and Xie, 2012). Thus, and apart from the recently introduced label-free systems (Kenakin, 2009; Scott and Peters, 2010), technologies currently established rely on fluorescence or luminescence reporter systems to access the different levels of GPCR signaling (Boehme and Beck-Sickinger, 2009), ranging from the ligand binding event (Zwier et al., 2010) to functional assays whereby second messenger accumulation, receptor desensitization or reporter activation is monitored in living cells (Eglen, 2005; Inoue et al., 2012; Zhang and Xie, 2012). Amongst the available assays, fluorescence detection of intracellular calcium following activation of Gq/11 coupled receptors...
constitutes a stamp for current HTS assays. Typically, recombinant cell lines are cultured on microtiter plates (96–1536-wells) and stained with calcium sensitive dyes that exhibit enhanced fluorescence upon calcium binding. Test compounds are then added to the cell system and the generated calcium transients are monitored in real time (Hodder et al., 2004; Marshall et al., 2006). Successful attempts to couple non-Gqα receptors (e.g., GαT or Gαi) by using cell lines expressing promiscuous Gq11 and Gαi1 proteins have also been demonstrated (Kostenis et al., 2005), which makes calcium mobilization assays attractive to probe a variety of GPCRs. However one major drawback of such functional screens is the dependency on external and expensive systems for signal acquisition like laser scanners, microscopes or charge coupled devices (CCD). Moreover, as miniaturization of cell-based assays for drug screening systems through microfluidics is gaining momentum (Martins et al., 2012), the demand for new detection strategies compatible with the low culture volumes and reduced cell numbers provided by such systems is increasing (Kamei et al., 2003).

Recently, the use of hydrogenated amorphous silicon (a-Si:H) photosensors for fluorescence, chemiluminescence and colorimetric detection of biomolecules have been reported (Conde et al., 2008). Applications include the detection of DNA sequences (Kamei et al., 2005) and DNA hybridization (Pimentel et al., 2008), antibody/antigen recognition (Pereira et al., 2011), detection of food toxins (Caputo et al., 2012; Novo et al., 2012), detection of green fluorescence protein expression (Joskowiak et al., 2011) and detection of intrinsic fluorophores in bacterial systems (Joskowiak et al., 2012). These sensors are characterized by high quantum efficiencies in the range of visible light and lower dark currents when compared to crystalline silicon (Kamei et al., 2003). Additionally, a-Si:H films are compatible with microfabrication techniques and thus easily integrated into microfluidic devices, opening up prospects for the fabrication of miniaturized monolithic devices with potential gains in assay sensitivity, reproducibility and time (Novo et al., 2012).

In this work, the feasibility of using a-Si:H photodiodes with integrated absorption filters to detect calcium dynamics inside living mammalian cells cultivated in vitro via the calcium-sensitive fluorescent dye Fluo-4 (Gee et al., 2000) is demonstrated. Intracellular calcium ($Ca^{2+}$) fluxes are generated by stimulating cultured HEK 293T cells with chemical compounds that either: (i) permeabilize cell membranes to $Ca^{2+}$ (ionomycin) or (ii) interact with the endogenous muscarinic M1 GPCR (Thomas and Smart, 2005) (carbachol and pirenzipine). The changes in fluorescence are detected using a-Si:H photodiodes with an integrated fluorescence filter and compared with those obtained by fluorescence microscopy.

2. Materials and methods

2.1. Chemicals and reagents

Fibronectin, carbachol and pirenzipine were purchased from Sigma. The calcium indicators Fluo-4 salt, Fluo-4 AM, Fluo-4 Direct and the reagents ionomycin calcium salt, probenecid, and calcium calibrated solutions were from Molecular Probes. Hank’s balanced solution (HBSS: 1.26 mM CaCl$_2$, 0.493 mM MgCl$_2$, 0.407 mM MgSO$_4$, 5.33 mM KCl, 137 mM NaCl, 0.338 mM Na$_2$HPO$_4$, 5.5 mM D-glucose), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 × HEPEs) and cell culture reagents were purchased from Gibco. Sylgard™ 184 polydimethyl siloxane (PDMS) and the corresponding curing agent were purchased from Dow Corning. Human Embryonic kidney (HEK) 293T cell line was from American type culture collection, ATCC (ATCC® CRL-11268).

2.2. Measurement apparatus

2.2.1. Microscopy

Microscopy studies were performed using an inverted Olympus CKX41 fluorescence microscope equipped with a mercury arc lamp HBO 50 W (OSRAM) for illumination and band-pass filters in the range of 460–490 nm. Images were acquired with a Plan CN 10 x objective. Real-time imaging recording was performed using the XC30 CCD camera and the acquisition software cellsens$^{\text{TM}}$ from Olympus. Exposure time and gain were set to 1 s and 6 db, respectively, and real-time recordings were acquired for 250 s at a rate of 1 frame/s.

2.2.2. Amorphous silicon (a-Si:H) photodiodes with integrated fluorescence filter

The a-Si:H p-i-n photodiodes with integrated fluorescence filters were fabricated from glass substrates (AF45, Schott). Fabrication started with the deposition of a 2 μm thick amorphous silicon carbon alloy film (a-SiC:H) as the optical absorption filter by radio frequency plasma enhanced chemical vapor deposition (RF-PECVD), using deposition temperature and pressure of 100 °C and 0.1 Torr, respectively. Silane (SiH$_4$) and ethylene (CH$_4$) gas flows were set to 9.9 and 0.9 sccm, respectively, in order to control the carbon content of the film and hence, its optical characteristics (Lipovsek et al., 2010). The transmittance properties of the filter were characterized by illuminating the sample from the glass side, using a tungsten–halogen lamp (250 W) coupled to a monochromator (McPherson 3035) for wavelength selection (650–400 nm). The sample was vertically aligned with a crystalline silicon (c-Si) photosensor with known responsivity (Advanced Photonics) and the generated current was quantified using a picocomparator (Keithley 237). A reference sample (bare glass) was used as control. The fabrication of the photodiodes proceeded with the deposition, by sputtering, of indium tin oxide (ITO) as the transparent, bottom electrode (1000 Å). The a-Si:H p-i-n layers were obtained by sequentially depositing 200 Å of p-doped a-Si:H, 5000 Å of intrinsic a-Si:H and 200 Å n-doped a-Si:H using RF-PECVD, at 250 °C and 0.1 Torr. Diborane (B$_2$H$_6$) and phosphine (PH$_3$) were the source gases for the p- and n-layer, respectively. The top contact consisted of 150 Å film of titanium tungsten (TiW) and 1000 Å of aluminum (Al), deposited by sputtering, using a physical mask to define an array of 48 contacts. Each contact presented an area of 2 mm$^2$ and constituted the actual sensing unit of the device (Fig. 1A). The optoelectronic properties of the device were characterized by evaluating the current density–voltage ($J$–$V$) response.

![Fig. 1.](A) Schematic representation of the cross-section of an a-Si:H p-i-n photodiode with integrated a-SiC:H fluorescence filter. (B) Configuration used to measure the fluorescence of calcium solutions and cell cultures showing a PDMS well aligned with the photodiode from the glass side.
the external quantum efficiency (EQE) in the range 400–650 nm and
detection limits (current density J, versus incident photon flux \(\Phi\)). The EQE was determined using Eq. (1) where \(J_{ph}\) is the
photocurrent density (A cm\(^{-2}\)), \(\Phi\) is the incident photon flux
(cm\(^{-2}\) s\(^{-1}\)), and \(q\) is the electron charge (1.60 \(\times 10^{-19}\) C)

\[
J_{ph} = \frac{q}{\Phi} \quad (1)
\]

The light source was either the tungsten–halogen lamp coupled
to the monochromator or a light emitting diode (LED), with a peak
emission wavelength at 470 nm, coupled to a 500 nm shortpass
filter (Thorlabs) as the excitation light. For the determination of
the photon flux \(\Phi\), the calibrated c-Si photosensor was used under
the same illumination conditions.

2.3. Fabrication of PDMS wells

PDMS wells were fabricated by pouring a mixture of the
polymer and curing agent at a 10:1 ratio into a Petri dish and
promoting curing for 2 h at 70 C. The cured PDMS was cut in
1 \(\times\) 1 cm\(^2\) (\(-1\) cm height) and punched in the center to form
0.5 cm diameter wells. 100 \(\mu\)m-thick PDMS sheets were fabricated by
spin-coating (Laurell Technologies) the polymer on cleaned
silicon wafers (WRS materials) and curing for 2 h at 70 C.
The sheets were cut in 1 \(\times\) 1 cm\(^2\) and used to seal the bottom of the
wells by performing corona discharge ionization (Electro-Techni-
que Products, Corona tester BD 20-AC) for 20 s.

2.4. Fluorescence and photocurrent measurements of
calcium-calibrated solutions

Fifty microliters of solutions containing defined concentrations of
calcium ions (0–1.35 \(\mu\)M Ca\(^{2+}\)) were prepared from calcium
calibrated solutions (Molecular Probes) according to the manufactu-
ers’ instructions and incubated with a 4 \(\mu\)M solution of Fluo-4
salt prepared in a Ca\(^{2+}\)-free buffer (10 mM K\(_2\)EGTA). The fluorescence
measurements were conducted on a Cary Eclipse spectrofluoro-
meter (Varian) equipped with a microplate stage. The photomulti-
plier tube (PMT) voltage was set to 600 V, and excitation and
emission filters were set to 490 and 516 nm, respectively, as required by
the optical characteristics of Fluo-4 in the presence of 1.35 \(\mu\)M Ca\(^{2+}\).

For the photocurrent measurements, PDMS wells containing
50 \(\mu\)L of each Ca\(^{2+}\) standard solution were aligned from the glass
side (Fig. 1B) with the photodiode. The LED was used as the
excitation light source. The measured current, \(i\), was acquired
using the picoammeter and converted to current density according
to the area of the photodiodes (2 mm\(^2\)). Signals were normalized
to the maximum value for comparison between the spectro-
fluorometer and photodiode measurement setups.

2.5. Cell culture and stimulation assays

HEK 293T cells (3 \(\times\) 10\(^6\) cells) were seeded in T75 cell culture
flasks with 10 mL of Dulbecco’s Modified Eagle’s medium (DMEM),
supplemented with 10% fetal bovine serum (FBS) and 1% anti-
biotic–antimycotic solution (penicillin, streptomycin and Fungi-
zone\(^{8}\)) and incubated at 37 C in a 5% CO\(_2\) atmosphere until
reaching 80% confluence (\(-4\) days). For microscopy monitoring of
stimulation assays, cells were detached by incubation with tryp-
sin–0.05% EDTA, seeded into cell-culture microtiter plates
(Becton–Dickinson) at a density of 50 \(\times\) 10\(^3\) cells/well (50 \(\mu\)L) and
allowed to adhere and spread for 16 h at 37 C in a 5% CO\(_2\)

atmosphere. The adhered cells were subsequently incubated for
30 min at 37 C with solutions of Fluo-4 Direct or 4 \(\mu\)M Fluo-4 AM
prepared in assay buffer (1X HBSS, 20 mM HEPES supplemented
with 2.5 mM probenecid) and then for 30 min at room
temperature, in the dark. Test compounds (ionomycin, carbachol
and pirenzepine) were prepared fresh, and diluted in assay buffer
in such a way as to provide effective concentrations inside the
wells of the order of 10\(^{-3}\)–10\(^{-8}\) M for ionomycin and 10\(^{-2}\)–
10\(^{-8}\) M for carbachol and pirenzepine. The microtiter plate was
mounted on the microscope stage and the baseline fluorescence
was acquired for 20–50 s. Addition of test compounds (20 \(\mu\)L)
was performed manually with the aid of a pipette and the change in
fluorescence was monitored throughout the following 200 s. In the
pirenzepine assay, fluorescence was monitored for 5 min after
addition of pirenzepine. Carbachol (EC\(_{50}\)) was then added and
fluorescence signals were monitored for 200 s. Negative controls
were performed by injecting 20 \(\mu\)L of assay buffer.

For the photodiode monitoring of stimulation assays, PDMS
wells were first treated with 100 \(\mu\)g/mL of fibronectin for 1 h, at
37 C. The wells were rinsed with phosphate buffer and kept at
4 C until further use. On the day of the stimulation assays, cells
were seeded on the PDMS wells (75 \(\times\) 10\(^3\) cells/well in a total
volume of 50 \(\mu\)L) and allowed to adhere and spread for 1 h, at
37 C, under a 5% CO\(_2\) atmosphere. Incubation with the calcium
sensitive dyes was performed as described above. The cell-
containing PDMS wells were then aligned with the photodiodes
from the glass side (Fig. 1B), and illuminated vertically using the
LED coupled to a 500 nm shortpass filter. The baseline signal
was acquired for 20–50 s using the picoammeter at 0 voltage bias. Test
compounds (prepared as described above) were injected manually
(20 \(\mu\)L) into the wells and the change in the photodiode’s current
was monitored for the following 200 s.

2.6. Data analysis

Time dependent light intensity was extracted from fluores-
cence real-time images (1 frame/s) using the ImageJ (NIH). An in-house software was developed (Labview) for real-
time current acquisition. A GPIB connection ensured communica-
tion with the Keithley picoammeter. Data were recorded every
second as follows: 8 data points were acquired regularly with a
10 ms integration time. The averaged value was then sent to the
computer. Under the illumination light conditions, the photodiode
time response was measured in the 1–10 s range. Therefore, the
software acquisition method was not considered as a limitation for
the experiments. In the cell stimulation assays, the signals acquired
in the first 20–50 s (prior to compound injection) were averaged
to obtain a baseline signal (S\(_0\)) and the response signals
(S) were normalized according to (S/S\(_0\)). Normalized responses
were extracted as a function of the concentration of the stimulat-
ing compound and fit to a four parameter Hill dose response curve
(no constraints), according to the following equation using the
software GraphPad Prism\(^{8}\):

\[
y = \frac{A1 + (A2\text{-}A1)}{1 + 10^{\log(Ec50-S)\text{-}x\text{-}p}}
\]

where \(y\) is the response signal, A1 is the bottom asymptote
(minimum response), A2 is the top asymptote (maximum response),
x is the compound concentration, LogEC50 is the logarithm of
the effective concentration which generates the
half-maximum response and \(p\) is the Hill slope. In order to
calculate the confidence, we performed a Fisher test for model comparison
and unpaired T tests (GraphPad Prism\(^{8}\)) for individual coefficient
comparison. Signal to noise ratio (S/N) was determined according
to Zhang et al. (1999).
3. Results and discussion

This work investigates the feasibility of using a-Si:H photodiodes integrated with absorption filters to detect calcium dynamics inside living mammalian cells cultivated in vitro via fluorescence monitoring.

3.1. Characterization of the a-Si:H p-i-n photodiodes with integrated fluorescence filter

A major challenge in fluorescence detection relies on the ability to discriminate between excitation and emission photons, while assuring that a maximum number of emission photons reach the detector. Previous work from our group has demonstrated the applicability of a-Si:C:H alloy films as optical absorption filters for integrated fluorescence detection (Jokowiak et al., 2012). In this work, an a-Si:C:H film with a carbon content of 8% (Lipovsek et al., 2010) was fabricated and integrated with a-Si:H p-i-n photodiodes. Excitation and emission spectra of Fluo-4 and the transmittance of the filter as a function of wavelength are depicted in Fig. S1 in supplementary data.

The efficiency of the photodiodes was characterized by determining the external quantum efficiency (EQE), a parameter that reflects the number of detected electrons as a function of incident photons (Pimentel et al., 2008). Fig. 2A shows the calculated EQE and the transmittance of the filter at relevant wavelengths. The filter presents low transmittance (≪0.001%), and hence high absorption, for wavelengths below 480 nm, becoming increasingly transparent as wavelength increases. At 490 nm (λEx of Fluo-4), only 0.013% of the photons are transmitted, whereas at 516 nm (λEm of Fluo-4), transmittance is ~30 times higher. The EQE increases up to 600 nm as the number of photons impinging on the device increase (increased transmittance). The EQE at 516 nm is approximately 20 times higher than the EQE at 490 nm. Additionally, in the context of fluorescence detection and due to the characteristic broad bell-shaped emission spectrum of organic fluorophores such as Fluo-4, further gains in current are expected by collecting a fraction of the lower energy photons that are emitted at the higher wavelengths. For the higher wavelengths, the observed decrease in EQE can be explained by a decrease in absorption in the a-Si:H layers that constitute the p-i-n junction due to the characteristic a-Si:H energy band gap (Eg ~1.7 eV) (Kamei et al., 2003).

The J-V response of different photodiodes, both in the dark and under illumination, was characterized and a representative J-V curve is shown in Fig. S2, supplementary data. At 0 V bias, the dark current density is as low as 0.1 nA cm⁻². Incident monochromatic light at 490 nm (490_mch) induces a 100 fold increase in the photodiode current density (∼40 nA cm⁻²), which is related to the transmittance of the filter at this wavelength. Still, the current generated at 526 nm is approximately 20 times higher in good agreement with the EQE values calculated above. At this point, the possibility of using a broadband LED, with a peak emission wavelength at 470 nm as the excitation light source was tested. Due to the characteristic spread around an LED peak wavelength (typically 40–90 nm) (Dandin et al., 2007), the LED was coupled to a shortpass filter in order to cut wavelengths above 500 nm. The power density of the incident LED light was calculated to be 6 mW cm⁻² which results from 1.3 × 10¹⁶ cm⁻² s⁻¹ photon flux. These values contrast with the power density and photon fluxes obtained at 490_mch nm ([0.25 mW cm⁻² and 6.0 × 10¹⁴ cm⁻² s⁻¹, respectively]. However, at 0 V, the current generated by the LED on the p-i-n photodiode is 5 nA cm⁻², thus a smaller current is generated with a 20× increase in the photon flux when compared to 490_mch nm (Fig. 2B and Fig. S2 in supplementary data). This trend is explained by the low transmittance of the filter at 470 nm (< 0.001%) and the consequent reduced EQE values at this LED wavelengths. Fig. 2B also shows that the current density at λEm of Fluo-4 varies linearly within a wide range of photon fluxes (10¹⁰–10¹⁴ cm⁻² s⁻¹), and that current intensities as low as 4 pA can be detected. Using the LED as the incident light source and due to the absorption proprieties of the integrated filter, improved ratios between JLED and Jdark for higher values of Φ can be achieved. In view of this, the LED was selected as the excitation light source for the biological experiments.

3.2. Fluorescent detection of calcium ions in solution using the p-i-n photodiodes with an integrated fluorescence filter

The feasibility of using the a-Si:H photodiodes to detect calcium in combination with a fluorescent indicator was assessed first using calibrated calcium buffers. These buffers consist of standards of CaEGTA complexes that in equilibrium produce solutions of Ca²⁺ ions with defined concentrations that span the expected physiological range. These Ca²⁺ ions are free to react with the Fluo-4 salt, producing a fluorescence signal that is proportional to the Ca⁻²⁺ concentration (Takahashi et al., 1999). Cells at rest have an iCa²⁺ concentration of 100 nM that can increase up to 10 times upon a stimulus (Berridge et al., 2000). Thus, standard Ca²⁺ solutions were prepared in the 0–135 μM concentration range (0, 0.017, 0.038, 0.1, 0.35, 0.6 and 1.35 μM). Fig. 3 compares the signal intensities obtained with the Fluo-4 stained cells using both the calibrated calcium solutions and a standard spectrophotometer (open squares). Data were normalized to the maximum value to facilitate comparison. Results show that the intensities of the output signals from both systems increase as Ca²⁺ concentration increases. Background signals are slightly higher with the p-i-n...
photodiode setup, mostly due to the contribution of incident photons to the generated current. This background signal is reduced in the spectrofluorometer plate reader due to the decoupling angle (90°) between the excitation beam and the emission channel. For this reason, the sensitivity as given by the inverse slope is also improved in the spectrofluorometer read-out. Nevertheless, the current densities of the p-i-n photodiodes varied approximately one order of magnitude (2 × 10^-8–2.6 × 10^-7 A cm^-2), throughout the analyzed calcium concentration range, making their performance sufficient to be tested for iCa\(^{2+}\) monitoring.

3.3. Monitoring intracellular calcium dynamics using p-i-n photodiodes

As a first approach, HEK 293T cells were stimulated with different concentrations of ionomycin, an ionophore that renders the cell membranes permeable to Ca\(^{2+}\). Since the extracellular calcium concentration is elevated with respect to the intracellular environment, stimulation with ionomycin leads to an increase in iCa\(^{2+}\), as a result of transport of calcium ions from both the extracellular space and intracellular stores (Erdahl et al., 1994; Vasilev et al., in press).

In these studies cells were loaded with the ester form of Fluo-4 (Fluo-4-AM) which is cell permeable. Two different formulations of Fluo-4-AM were tested: a Fluo-4-AM solution and Fluo-4-Direct. (Fluo-4-AM) which is cell permeable. Two different formulations of Fluo-4-AM were tested: a Fluo-4-AM solution and Fluo-4-Direct.

![Fig. 3.](image) Comparison of signal intensities obtained when measuring Fluo-4-induced fluorescence of Ca\(^{2+}\) standards with photodiodes (closed squares) and a standard spectrofluorometer (open squares). Data were normalized to the maximum value to facilitate comparison between the distinct setups (Sj/Smax).

![Fig. 4.](image) (A) Representative real-time signals of calcium fluxes obtained using the microscope (diamonds) and the p-i-n a-Si:H device (circles) in response to 5 μM of ionomycin (closed symbols) and assay buffer (open symbols). (B) Dose response curves for ionomycin stimulation assays. Data represent averages and standard error of the mean (SEM). Diamonds: Microscopy data with y = 2.86 × (98.6 – 2.86)/(1 + 10^6 × 0.27 × 8.72). n = 3 independent assays. Circles: integrated devices with y = 9.41 – (97.8 – 9.41)/1 + 10^(-6.31 × 8.72); n = 4 independent assays.
The possibility of using the integrated-filter p-i-n photodiodes for the characterization of GPCR signaling was investigated. The muscarinic type M1 receptor, which is endogenously expressed in the HEK 293T cell line, was chosen as the concept model (Thomas and Smart, 2005). In the intracellular environment, M1 is commonly coupled to the Gqα11 type G-protein, which activates phospholipase C (PLC). Subsequently, IP3 is generated triggering the release of Ca\(^{2+}\) from the endoplasmic reticulum into the cytoplasm (Karakiulakis and Roth, 2012). In general, the iCa\(^{2+}\) fluxes are transient, characterized by a fast peak rise in iCa\(^{2+}\), followed by a slow decay as desensitization of the GPCR occurs and the iCa\(^{2+}\) concentration is restored (Video 1 in supplementary data). Results shown in Fig. 5A represent a characteristic real-time analysis of HEK 293T cells stimulated with 1 mM of carbachol (an M1 agonist), confirming that the expected iCa\(^{2+}\) kinetics could be observed with the integrated p-i-n photodiodes. For a 1 mM stimulus, the device’s current increased on average 28% with respect to the baseline (n=8 independent experiments), whereas negative controls (n=6 independent experiments), contributed on average to a 3.5% increase in the device’s current. Higher increments over the baseline were observed using the fluorescence microscope setup, once again reflecting the different optical filtering capabilities (interference filter in the microscope) of the incident photons. Consequently, the S/N ratio is larger in the microscope (S/N=30) when compared to the p-i-n photodiode setup (S/N=7.5). Fig. 5B shows the dose-response curves of carbachol obtained using both measurements setups. The LogEC\(_{50}\) was determined to be \(-4.98 \pm 0.2\) with the integrated device and \(-4.95 \pm 0.10\) with the microscope setup, which translates into EC\(_{50}\) values of 105 and 111 nM, respectively. These values are in good agreement with the EC\(_{50}\) values (4–11 nM) reported by other authors for HEK 293T endogenous M1 (Conklin et al., 1992; Mundell and Benovic, 2000). No significant statistical differences were observed between the different setups with regards to the calculated values of LogEC\(_{50}\) (p=0.4) and the generated curves (p=0.93). Values of EC\(_{50}\) in the nanomolar range have been reported when using recombinant cell lines that overexpress the M1 GPCR (Liu et al., 2010). However, since in these recombinant cell systems the expression of cell surface receptors is increased over native cells, higher signals are typically obtained for lower compound dosage.

Finally, the specificity of the cell response observed upon carbachol stimulation was analyzed. The rationale behind this analysis is based on the fact that multiple receptor families are expressed in the HEK 293T cell line. Thus, agonist cross-reactivity among the different receptor families as well as endogenous ion-gated channels can contribute to calcium signaling independently from M1 activation. Pirenzepine, a well-known neutral antagonist of the M1 receptor with sub-type specificity (Piggott et al., 2002) was used in this study. The addition of pirenzepine to the cell culture is expected to specifically block M1 activation upon agonist addition in a dose response manner. Fig. 5B shows the dependency of the response signal as a function of the pirenzepine concentration in the cell culture and upon addition of carbachol at an EC\(_{50}\) concentration (0.05 mM). Results obtained with the photodiodes and fluorescence microscope show that maximum responses are obtained in the absence of pirenzepine. As pirenzepine concentration increases, the signal decreases according to a typical inhibitory dose response curve. It is possible thus to conclude that the transient signals produced upon carbachol addition (Fig. 5B) are derived from specific M1 activation. Data from the response curves was used further to determine values of Log IC\(_{50}\). The Log of inhibitor concentration that reduces the maximum response by half. No significant differences (p=0.4) were observed between values obtained with the integrated p-i-n photodiodes...
(Log \( IC_{50} = -5.4 \pm 0.2, IC_{50} = 4.2 \mu M \)) and with the fluorescence microscope (Log \( IC_{50} = -5.3 \pm 0.2, IC_{50} = 4.9 \mu M \)).

4. Conclusions

The possibility of using a-Si:H photodiodes with integrated fluorescence filters for the real-time evaluation of drug-induced changes in the levels of second messengers inside live cells was demonstrated. The devices developed were able to detect changes in \( iCa^{2+} \) concentration and identify different \( iCa^{2+} \) dynamics such as sustained plateaus or transient fluxes. Furthermore, dose-response profiles for both agonism and antagonism were established with the same accuracy as provided by standard optical systems. Most importantly, the challenge of detecting endogenous GPCRs that are usually expressed at lower densities was consistently met. The uniqueness of the a-Si:H devices relies on their compatibility with microfabrication techniques, making it possible to envisage the fabrication of micron-sized sensors that can easily be integrated into miniaturized cell platforms for drug screening. In particular, integration with microfluidic platforms that enable automatic fluid handling is expected to improve assay reproducibility with major impacts on the current S/N ratio.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.08.037.

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