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Stepwise dosing protocol for increased throughput in label-free impedance-based GPCR Assays

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2   Stepwise Dosing Protocol for Increased Throughput in Label-Free Impedance-Based GPCR Assays
3
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33   **SUMMARY:**
34   This protocol demonstrates real-time recording of full dose-response relationships for agonist-
35   induced GPCR activation from a single cell layer grown on a single microelectrode using label-free
36   impedance measurements. The new dosing scheme significantly increases throughput without
37   loss in time resolution.
38

39   **ABSTRACT:**
40   Label-free impedance-based assays are increasingly used to non-invasively study ligand-induced
41   GPCR activation in cell culture experiments. The approach provides real-time cell monitoring with
42   a device-dependent time resolution down to several tens of milliseconds and it is highly
43   automated. However, when sample numbers get high (e.g., dose-response studies for various
44   different ligands), the cost for the disposable electrode arrays as well as the available time

45   resolution for sequential well-by-well recordings may become limiting. Therefore, we here
46   present a *serial* agonist addition protocol which has the potential to significantly increase the
47   output of label-free GPCR assays. Using the *serial* agonist addition protocol, a GPCR agonist is
48   added sequentially in increasing concentrations to a single cell layer while continuously
49   monitoring the sample's impedance (agonist mode). With this serial approach, it is now possible
50   to establish a full dose-response curve for a GPCR agonist from just one single cell layer. The serial
51   agonist addition protocol is applicable to different GPCR coupling types, G_q $G_{i/o}$ or G_s and it is
52   compatible with recombinant and endogenous expression levels of the receptor under study.
53   Receptor blocking by GPCR antagonists is assessable as well (antagonist mode).
54

INTRODUCTION:

55

56   This report presents a detailed description of a serial addition protocol developed for the
57   quantification of ligand-induced G protein-coupled receptor (GPCR) activation in adherently
58   grown cells by label-free impedance measurements. G protein-coupled receptors (GPCRs) are
59   involved in a multitude of physiological functions and human diseases[1]. Because of this and their
60   good accessibility at the cell surface, GPCRs are one of the most important drug targets. This
61   assessment is reflected in an estimated number of ~700 approved drugs targeting GPCRs,
62   equivalent to a ~35% share on all marketed drugs[2].
63

64   The development of new drugs comprises two central processes: (i) the identification and
65   functional characterization of biological target molecules, and (ii) the discovery of new lead
66   substances and their development into administrable drugs. In both processes, efficient methods
67   are required to quantitatively evaluate drug-target interactions and the subsequent biological
68   downstream response. Different stages of the pre-clinical drug development process make use
69   of different methods of analysis ranging from biomolecular interaction studies between drug and
70   target, over functional studies on cells in culture, to experiments on excised organ material or
71   whole animals. Both, physiological significance and biological complexity increase from the
72   former to the latter[3]. Although it is the overall goal to minimize animal experiments,
73   pharmacological studies using isolated organs from laboratory animals or even whole animals are
74   regarded inevitable to comprehensively characterize new drug candidates. In terms of analytical
75   readout, organ pharmacology studies provide a distal, integrative "holistic" functional response
76   of highest physiological relevance. A drawback of such experiments is that they are not
77   compatible with *high throughput screening* for technical as well as ethical reasons and have been
78   largely substituted by studies based on in vitro cell culture[4].
79

80   Methods to quantify GPCR activation in cell cultures include different label-based chemical
81   assays, which specifically detect second messengers, phosphorylation state of downstream
82   signaling proteins, transcriptional activation via certain transcription factors or ligand-induced
83   intracellular receptor trafficking[4,5]. A drawback of such label-based assays is the necessity to label
84   the cells with potentially harmful dyes or radioactive markers. This often requires running the
85   assay as an endpoint-determination for an exposure time that has to be specified *a priori*. Using
86   label-based endpoint assays suffers from this very limited and biased timing of the experiment
87   and the risk that chemical labels and probes may interfere with the regular cell physiology –
88   potentially unnoticed by the experimenter.

89

90   In recent years, label-free assays for monitoring GPCR activation have emerged, like impedance-
91   based techniques or optical methods applying resonant waveguide gratings[5,6]. Labelling of the
92   cells is experimentally not required with these approaches. As these physical readouts operate
93   on low amplitude signals, such methods are considered non-invasive, they allow for continuous
94   cell monitoring potentially in real time and the observation time is only limited by the cell culture
95   not by the readout. Similar to readouts from whole organs, label-free approaches commonly
96   report on *holistic* cell responses, far downstream of receptor activation when integration along
97   the entire signaling network leads to time-dependent but rather unspecific changes in cell
98   morphology or mass redistribution. Whereas impedance-based assays measure the dielectric
99   signature of changes in cell shape[7,8], measurements using resonant waveguide gratings are
100   sensitive to changes in refractive index at the cell-substrate interface arising from *dynamic mass*
101   *redistribution* (DMR)[9]. The integrative character makes label-free methods extremely sensitive to
102   receptor-mediated events regardless of the type(s) of G protein (G_q, $G_{i/0}$, G_s, $G_{12/13}$) or β-arrestin
103   involved in the signaling cascade[6] and well-suited for endogenous expression levels of the
104   receptor.

105

106   In a standard label-free impedance-based assay the cells are adherently grown in multi-well
107   plates with coplanar gold-film electrodes deposited on the bottom of each well[10]. These electrode
108   arrays are connected to an impedance analyzer and the cell responses to an experimental
109   stimulus are recorded from individual wells by time-resolved impedance readings. In a typical
110   GPCR assay a ligand is added in individually different concentrations to each individual well. The
111   ligand-induced changes in the impedance time course are then analyzed with respect to
112   characteristic curve features such as maximum signal change, area under the curve, signal change
113   within a given time interval or slope of the curve at a specified time point, in order to quantify
114   the ligand's potency and efficacy[11].

115

116   The cost of the electrode arrays may limit the application of this technique in *high throughput*
117   *screening* (HTS) campaigns. Moreover, with increasing numbers of samples to be followed in
118   parallel, the number of individual measurements increases and thereby reduces the available
119   time resolution for each well gradually - even for state of the art multichannel recordings. Under
120   such conditions fast and transient cell responses may escape the measurement. In addition, the
121   conventional *one well – one concentration* approach imposes a significant time and cost factor
122   on perfused organ-on-chip or body-on-chip developments with respect to their suitability in
123   ligand-GPCR interaction analysis.

124

125   For this reason, we developed a stepwise dosing protocol that enables recording of full dose-
126   response curves of ligand-induced GPCR activation in cultured cell monolayers by continuously
127   monitoring the impedance of a single well while the agonist concentration is increased stepwise.
128   The *serial agonist addition* protocol significantly increases throughput per well from one
129   concentration to 10 or more concentrations, as shown on the current example of human
130   U-373 MG cells, which endogenously express the histamine 1 receptor (H1R). Thus, the method
131   has the potential to significantly improve throughput in label-free dose-response studies, while
132   the time resolution is retained at the instrumental maximum.

133
134 **PROTOCOL:**
135
136 **1.   Cell seeding on electrode arrays**
137
138 NOTE: Selection of electrode layout is a trade-off between sensitivity and number of cells under
139 study. The smaller the electrode, the more sensitive is the measurement but the smaller is the
140 number of cells under study. For cells showing strong impedance fluctuations over time under
141 baseline conditions, bigger or interdigitated electrodes are preferable.
142
143 1.1.   Pre-warm all solutions needed for standard cell passaging and seeding in a 37 °C water
144 bath. For assays with human U-373 MG cells it takes: phosphate buffered saline (PBS) without
145 calcium and magnesium, 0.05% (w/v) trypsin, cell culture medium (Eagle's Minimum Essential
146 Medium (EMEM) supplemented with 5% (v/v) fetal calf serum (FCS), 2 mM L-glutamine and
147 100 µg/mL penicillin/streptomycin).
148
149 1.2.   Rinse the cell layer of the stock culture grown on the bottom of conventional cell culture
150 flasks or dishes with PBS twice.
151
152 1.3.   Remove the PBS, add the trypsin solution (1 mL for 25 cm^2) and let the cells incubate at
153 37 °C for 5 min (applies to U-373 MG cells).
154
155 1.4.   Control for complete detachment of the cells from the bottom of the growth substrate by
156 microscopy.
157
158 1.5.   Stop the trypsin reaction as soon as the cells are completely detached by adding 9 mL of
159 cell culture medium per 1 mL of trypsin to the cell suspension. Carefully rinse remaining cells from
160 the bottom of the cell culture substrate by pipetting the suspension over the substrate.
161
162 1.6.   Collect the cell suspension with a pipette and transfer it to a centrifugation tube (15 mL
163 or 50 mL tube).
164
165 1.7.   Spin down the cells by centrifugation at 110 x *g* for 10 min at room temperature.
166
167 1.8.   Carefully remove the supernatant and thoroughly re-suspend the cell pellet in culture
168 medium before counting the cells (e.g. by using a hemacytometer for phase contrast microscopes
169 and manual counting).
170
171 1.9.   Adjust the cell suspension to the desired cell density. For experiments with U-373 MG
172 cells, use 100 000 cells/cm² to grow a confluent cell layer within 48 h. This translates to a cell
173 density of 200 000 cells/mL for 8-well electrode arrays with a growth area of ~0.8 cm² and a well
174 volume of 400 µL.
175
176 NOTE: For reproducible GPCR activation experiments, cells should be grown to confluent

177 monolayers on the electrode arrays. To ensure proper receptor expression on the cell surface,
178 cells should be seeded at least 36 h before performing the experiment. Testing different cell
179 densities for cell seeding is often meaningful to identify the best experimental conditions.
180
181 1.10.   Add the cell suspension to the wells of the electrode array and let the sells settle down at
182 room temperature for 10 – 15 min in order to ensure homogenous distribution of cells on the
183 bottom of the wells.
184
185 1.11.   Grow cells for **at least** 36 h in a standard cell culture incubator with 5% CO_2 (dependent
186 on medium type) at 37 °C and humidified atmosphere. Change cell culture medium 24 h prior to
187 the experiment.
188
189 1.12.   On the day of the experiment, inspect the cell layers on the electrode arrays by (phase
190 contrast) microscopy to ensure complete coverage of electrodes with cells.
191
192 **2.   Equilibration of cells in serum-free medium**
193
194 2.1.   Pre-warm serum-free medium, in this study: Leibovitz's L15 medium.
195
196 2.2.   Remove cell culture medium from cells grown on electrode arrays and replace by pre-
197 warmed serum-free medium. Use 200 µL for 8-well format electrode arrays, and 150 µL for 96-
198 well electrode arrays.
199
200 2.3.   Let the cells equilibrate in serum-free medium at 37 °C for at least 2 h. The equilibration
201 time strongly depends on the cell type. For example, U-373 MG cells need 2 h, CHO cells need
202 4 h, BAEC may require overnight equilibration in L-15 medium.
203
204 NOTE: L-15 medium is CO_2 independent and requires a CO_2-free atmosphere. For equilibration in
205 L-15 set the incubator to 0 % CO_2. Equilibration can be monitored by impedance readings, which
206 we recommend to do for the first experiments.
207
208 **3.   Monitoring cell equilibration with impedance readings**
209
210 3.1.   Place the electrode array into the connecting array holder of the impedance analyzer.
211
212 3.2.   Ensure proper low impedance contact between electrodes and impedance analyzer. This
213 check is individually different for different instruments.
214
215 NOTE: If the instrument fails to make connection to the electrodes, open the contact clamp again,
216 readjust the electrode array for proper positioning inside the holder and retry.
217
218 3.3.   Select electrode type and/or multi-well format from the user interface of the software.
219
220 3.4.   Set up the measurement parameters. Different options are available.

221

222 NOTE: To select the most sensitive AC frequency, we refer to the literature and instrument
223 manuals as it depends on electrode layout and cell type under study. Typically, the sensing
224 frequency is in the range of 4 kHz – 50 kHz. Here, U-373 MG cells were grown on circular working
225 electrodes of 250 µm diameter and they were monitored at an AC frequency of 12 kHz.

226

227 3.4.1.   If single and multiple frequency data acquisition modes are available, select the single
228 frequency mode to ensure maximum time resolution. The measurement will be performed at this
229 single frequency. For the most widely spread instruments, there is a number of pre-set
230 frequencies along the available frequency window.

231

232 3.4.2.   If the number of wells under study is low or time resolution is not critical, select multiple
233 frequency recordings instead. Impedance readings at a specified number of frequencies will be
234 recorded for every well for later in-depth analysis.

235

236 NOTE: The time resolution decreases with increasing number of frequencies recorded per well
237 and increasing number of wells. The options for frequency and data acquisition mode selection
238 vary with instrument type and version.

239

240 3.5.   Start acquisition of time course data.

241

242 3.6.   Follow the cell layer impedance (at least 2 h) until the impedance has stabilized. In the
243 meantime, prepare the agonist solutions.

244

245 3.7.   When the cell layers have reached a stable impedance level, either (i) proceed to the serial
246 agonist addition within the same experiment or (ii) terminate data acquisition and start a new
247 dataset for monitoring agonist-induced receptor activation.

248

249 **4.   Preparation of agonist solutions for experiments in agonist mode**

250

251 4.1.   Calculate the concentration of agonist solutions as needed for each step of serial dosing
252 according to equation (1). n ranges from 1 to the total number of serial additions i. x denotes
253 concentration and volume in the well at step n. y denotes concentration and volume of the
254 "solution-to-be-added" in step n.

255

256 $$c_{yn} = \frac{(c_{xn+yn} \times V_{xn+yn}) - (c_{xn} \times V_{xn})}{V_{yn}}$$ (1)

257

258

259 NOTE: Consider the number of replicates and calculate the total volume of "solution-to-be-
260 added" for each concentration step. Results of a typical calculation are given in **Tables 1 – 4**. It
261 takes a general idea about the agonist concentration range to be studied as the range defines the
262 concentrations and number of portions to be administered during serial addition. Using the serial
263 agonist addition protocol the agonist concentration is increased stepwise. Therefore, the amount

264 of agonist already in the well when the next dose is added has to be taken into account. When
265 the *number* of agonist molecules already present in the well is $n_x = c_x \cdot V_x$ (with the current
266 concentration c_x and volume V_x) and the number of molecules in the well after the next addition
267 is n_{x+y}, the number of molecules to be added n_y is determined by the concentration c_y and volume
268 V_y of the solution to be applied to the well ($n_y = c_y \cdot V_y$). After adding a portion of agonist, the new
269 amount of agonist molecules in the well is: $c_{x+y} \cdot V_{x+y} = c_x \cdot V_x + c_y \cdot V_y$. This calculation applies for
270 each subsequent step. Because of the interdependence of agonist concentration in the well and
271 the amount of agonist in the portions to be added with each step, it is important to define final
272 concentrations after each step in advance.
273
274 **Mode 1:** The volume in the well will increase with each step as liquid is continuously added.
275 Using this mode and an 8well format, use V_{x1} = 200 µL and $V_{y1\,...}\,V_{yi}$ = 30 µL.
276
277 **Mode 2:** The volume in the well is constant as the volume added with each step is removed just
278 before the subsequent addition. Using this mode and a 96 well format, use V_{x1} = 150 µL and V_{y1}
279 $_{...}\,V_{yi}$ = 75 µL.
280
281 4.2.   Print the data sheet with the total volume per concentration and detailed instructions for
282 pipetting.
283
284 4.3.   Prepare all solutions in the required amount. Make all agonist solutions in the same
285 serum-free medium as used for equilibration of the cells.
286
287 CAUTION: Histamine dihydrochloride is considered hazardous by the 2012 OSHA Hazard
288 Communication Standard (29 CFR 1910.1200). Histamine causes skin irritation, serious eye
289 irritation, may cause an allergic skin reaction, allergy, asthma symptoms or breathing difficulties
290 if inhaled and may cause respiratory irritation. Please consider the safety data sheet.
291
292 NOTE: Make agonist solutions as fresh as possible. Stability of agonists in solution may vary
293 considerably. Store solutions at 4 °C or below until use in the experiment. For some molecules
294 additional stabilizing additives, like BSA when using peptide-based or lipid-based molecules, may
295 be considered to prevent adsorption to the walls of wells and tubes.
296
297 4.4.   If performing the experiment in 96-well format, transfer solutions to a conventional 96-
298 well plate (no electrodes) and use an 8- (or 12-) channel pipet for quick liquid transfer to the
299 electrode array.
300
301 **5.   Preparation of agonist solutions for experiment in antagonist mode**
302
303 NOTE: Prepare the antagonist solution(s) with the concentration to be applied throughout.
304 Volume and concentration of antagonist solution depend on the mode of agonist addition (1 or
305 2). Examples for experiments in 8-well or 96-well format in addition mode 2: (A) 8-well format
306 (V_{x1} = 200 µL, $V_{Antagonist}$ = 200 µL); (B) 96-well format (V_{x1} = 150 µL, $V_{Antagonist}$ = 75 µL).
307

308 5.1.　　Calculate the concentration of each agonist solution needed for each step of serial dosing
309 as described in step 4.1.

310

311 5.2.　　Make all agonist solutions in the same serum-free medium as used for equilibration of the
312 cells and add the antagonist at the same final concentration as planned for the respective wells
313 in the experiment.

314

315 NOTE: In this case the histamine stock solution (10 mM) is prepared in L-15 medium. When
316 agonists are dissolved in other solvents (e.g., dimethyl sulfoxid (DMSO), ethanol) a solvent control
317 should be included to account for the increasing solvent load with each step of addition.

318

319 **6.　　　Performing the serial addition protocol in agonist mode**

320

321 6.1.　　Start data acquisition as described in steps 3.1 – 3.5.

322

323 6.2.　　Pre-warm the agonist solutions prior to use by placing them in the incubator about 10 –
324 15 min before addition.

325

326 NOTE: When using thermo-labile substances, the solutions should not be kept at 37 °C for too
327 long. If pre-warming for 10 – 15 min is considered critical, bring solutions to 37 °C shortly before
328 addition in a water bath.

329

330 6.3.　　Run the agonist serial dosing dependent on the selected addition mode. Following **mode
331 1** the total volume in the well increases with each addition of the next dose. In **mode 2** the same
332 volume which is added with each step is also removed again just before adding the next higher
333 dose.

334

335 NOTE: Time needed for equilibration of the cell layer in between two subsequent agonist doses
336 depends on the cells' response time. An initial experiment in parallel mode (one well – one
337 concentration) reveals (i) cell response times for different agonist concentrations and (ii) the most
338 sensitive curve parameter (e.g., impedance maximum, impedance after time x).

339

340 **A: Mode 1 / 8-well format**

341

342 6.3.1.　Add 30 µL of the first solution with the lowest concentration of agonist to the cells that
343 were equilibrated in 200 µL of serum-free medium.

344

345 6.3.2.　Let the cells respond and equilibrate for the pre-defined period of time (e.g., 15 min).

346

347 6.3.3.　Add 30 µL of the second solution with the next higher concentration.

348

349 6.3.4.　Repeat steps 6.3.1-6.3.3 with the third, fourth, and so on, agonist solution.

350

351 NOTE: Working with 10 concentration steps will result in a total volume of 500 µL in the end of

352   the experiment, which is just below the maximum applicable volume of these wells of ~ 550 µL.
353
354   **B: Mode 2 / 96-well format**
355
356   NOTE: Pause data acquisition during each liquid handling step (addition/removal) via the
357   impedance instrument's software when running experiments in 96-well format. The more
358   elaborate liquid handling may interfere with data acquisition. Use a multi-channel pipette.
359
360   6.3.5.   Pause data acquisition.
361
362   6.3.6.   Add 75 µL of the first solution with the lowest concentration of agonist to the cells that
363   were equilibrated in 150 µL of serum-free medium.
364
365   6.3.7.   Resume data acquisition.
366
367   6.3.8.   Let the cells respond and equilibrate for the pre-defined period of time (e.g., 15 min).
368
369   6.3.9.   About 1 – 2 min before the regular equilibration time ends, pause the measurement and
370   remove 75 µL from each well.
371
372   NOTE: The time point at which solution should be removed depends on the number of wells
373   monitored in parallel and the pipetting speed. Time needed for removal of the solutions should
374   not exceed the time between subsequent steps.
375
376   6.3.10. Add 75 µL of the second solution with the next higher concentration and resume the
377   measurement.
378
379   6.3.11. Repeat steps 6.3.8-6.3.10 with the third, fourth, and so on, agonist solution.
380
381   **7.   Performing the serial addition protocol in antagonist mode**
382
383   7.1.   Start the measurement as described in steps 3.1 – 3.5.
384
385   7.2.   During equilibration of the cell layers, prepare antagonist solution (e.g., 200 µL of 1.5 µM
386   diphenhydramine hydrochloride in L15 medium).
387
388   CAUTION: Diphenhydramine hydrochloride has potential acute health effects. It is harmful if
389   swallowed or inhaled, may cause eye and skin irritation. It may cause respiratory and digestive
390   tract irritation. Please consider the safety data sheet.
391
392   7.3.   Pre-warm the antagonist and agonist solutions by placing them in the incubator about 10
393   – 15 min before addition to the cell cultures (cf. 6.2). If antagonist-free wells are included in the
394   assay as well, also pre-warm serum-free medium.
395

396   7.4.   Add the antagonist solution to the designated wells. Let the cells equilibrate with
397   antagonist for 15 – 20 min. If antagonist-free wells are included, add the same volume of serum-
398   free medium to these wells.

399

400   7.5.   According to antagonist addition in **mode 2**, remove solution from the wells
401   (A)   8-well format (200 µL)
402   (B)   96-well format (75 µL)

403

404   7.6.   Run the agonist addition sequence as described in step 6.3.

405

406   **8.**   **Data export and analysis**

407

408   8.1.   Export data using the impedance instrument's software in order to convert all recorded
409   data from proprietary into common data formats (e.g., csv). This step allows for data re-
410   organization and presentation with other software packages.

411

412   8.2.   Load the csv-formatted data to scientific data analysis software.

413

414   8.3.   Normalize impedance values by subtracting the impedance of the last data point before
415   the **first** addition of agonist solution and by setting the time of addition to t = 0. Plot the time
416   course of normalized impedance.

417

418   8.4.   Plot the individual time courses and identify the maxima in impedance after each addition
419   step. Compose a data sheet with these values.

420

421   8.5.   Plot the values of maximum (or minimum, if applicable) impedance changes as a function
422   of agonist concentration. This can be done for individual wells or for the averages (mean ± SD).

423

424   8.6.   Use a data fitting routine to determine half-maximal effective concentrations (EC_{50}) and
425   maximum response (E_{Max}) by using the four parameter logistic model (equation 2):

426

$$f(c) = A_2 + \frac{(A_1 - A_2)}{(1 + \left(\frac{c}{EC_{50}}\right)^n)} \qquad (2)$$

427   NOTE: c indicates the agonist concentration, A_1 is the maximum and A_2 is the minimum
428   asymptote of the sigmoidal dose-response curve ($A_1 = E_{Max}$). EC_{50} is the concentration at the
429   inflection point of the curve, and n corresponds to the Hill slope.

430

431

432   **REPRESENTATIVE RESULTS:**
433   A typical scheme for preparing the various agonist solutions is shown for an experiment using 8-
434   well electrode arrays with histamine as agonist in **Tables 1 - 4**. **Table 1** and **Table 2** present
435   volumes and concentrations for an experiment using addition **mode 1** (cf., **Figure 1**), while **Table**
436   **3** and **Table 4** present volumes and concentrations for an experiment following addition **mode 2**

437 (cf., **Figure 1**). Data have been calculated using equation (1).

438

439 A representative experiment is shown in **Figure 2** for confluent layers of U-373 MG cells, which
440 endogenously express the histamine 1 receptor, grown on an 8-well electrode array with a
441 working electrode of 250 µm diameter using histamine as agonist. The measurement was
442 performed at a single frequency of 12 kHz. Addition of the agonist concentration series was
443 performed following **mode 1**. In the selected experiment 10 solutions with increasing histamine
444 concentration (prepared according to step 4) were added sequentially every 15 min (**Figure 2A**).
445 The serial dosing scheme was applied to seven of eight wells. In one well agonist-free medium (L-
446 15) was added sequentially as a negative control in nine of ten steps. The last addition to this
447 control was a histamine solution providing a final concentration of 100 µM histamine in the well.
448 Data has been plotted with data analysis software and is shown as impedance change $\Delta|Z|_{12\,kHz}$
449 / kΩ along the experiment, with the impedance change and the time point of the first addition
450 being set to zero. The initial equilibration time in the experimental buffer of 2 h is not shown in
451 this plot. The time points of agonist addition are indicated by arrows.

452

453 The change in impedance relative to the baseline after each concentration step was extracted
454 from the curves using a data cursor. At steps with no clear maximum, the last data point before
455 the next addition was used for analysis. Impedance changes $\Delta|Z|_{12\,kHz}$ were plotted as a function
456 of histamine concentration (c_{x+y}) (**Figure 2B**, symbols). The transfer function of a four parameter
457 logistic model (cf. step 8.6) was fitted to the experimental data points from seven wells
458 (**Figure 2B**, lines). The fit results are shown in **Table 5**. **Figure 2C** and **Figure 2D** show the result of
459 averaging the data in **Figure 2A** and **Figure 2B**, presenting mean ± SD for seven wells. Average
460 time course data is summarized in **Figure 2C**, whereas the average dose-response curve is
461 provided in **Figure 2D**.

462

463 In dose-response relationships as shown in **Figure 2B,D**, the fitting procedure was applied to the
464 entire concentration range returning $EC_{50}=$ (0.86 ± 0.13) µM. The impedance response increases
465 monotonically with increasing histamine concentration except for the last two concentration
466 steps (30 µM, 100 µM final concentration). This response is presumably explained by
467 downregulation of the histamine receptor, desensitization or over-stimulation of the cells (see
468 discussion). To account for this effect, the data range for analysis has been reduced, as shown for
469 one single serial dosing experiment (**Figure 3**; well 1) selected from the data shown in **Figure 2**.
470 Moreover, setting the lower asymptote (A_2) to zero during least square optimization assuming no
471 impedance change in response to 0 µM histamine is well justified. Applying this three parameter
472 optimization provides an EC_{50} of (0.75 ± 0.12) µM (cf., **Figure 3B**). EC_{50} values were found to be
473 insensitive for either of the data analysis modes and were not significantly different.

474

475 Typical results for a 96-well plate experiment are summarized in **Figure 4**. U-373 MG cells were
476 grown on 96-well electrode arrays as described above. 32 wells were included in the
477 measurement. 8 wells served as agonist-free controls, exposed to medium only. 24 wells were
478 subjected to a series of increasing histamine concentrations calculated and prepared for addition
479 mode 2. The time course of impedance change is shown for all 32 individual wells in **Figure 4A**.
480 One of the curves (highlighted in red) shows an unusual time course and was excluded from

481   further analysis. Accordingly, data from 23 wells were averaged and plotted as time course of
482   average impedance change (mean ± SD) in **Figure 4B**. Each individual curve was analyzed as
483   described above for the dose-response relationship. Data points were averaged and plotted as
484   function of the final histamine concentration (**Figure 4C**). Dose-response data were analyzed by
485   means of the four parameter logistic model, which returned an average EC_{50} value of (1.0 ± 0.3)
486   µM.
487
488   A typical result for the serial agonist addition protocol in **antagonist mode** is shown in **Figure 5**.
489   Here, one well was pre-treated with 0.75 µM of the histamine receptor antagonist
490   diphenhydramine (red curve) 20 min before the first histamine was added (**Figure 5A**). The
491   control received antagonist-free medium (blue curve). The antagonist was added following **mode**
492   **2**, which means that 200 µL of the finally 400 µL were removed before the agonist series was
493   applied. The agonist (histamine) was added following serial dosing **mode 1** as described in the
494   examples above. It is important for experiments in antagonist mode that the antagonist
495   concentration is kept constant over the entire time course of the experiment. Accordingly, all
496   agonist solutions added to the well must also contain the pre-defined antagonist concentration
497   (in this case, 0.75 µM), while the control remains antagonist-free. An antagonistic effect becomes
498   apparent by a "delayed" increase of impedance within the serial dosing scheme corresponding
499   to higher agonist concentrations needed to induce a cell response. In the dose-response
500   relationship the effect of the antagonist is expressed as a rightward shift of the curves, which
501   corresponds to an increase in EC_{50}, provided the antagonist is a competitive ligand relative to the
502   agonist (**Figure 5B**). The EC_{50} was determined to (0.92 ± 0.05) µM in absence of the antagonist
503   and (14.7 ± 0.6) µM in its presence.
504
505   **FIGURE AND TABLE LEGENDS:**
506   **Figure 1: Serial agonist addition modes.** (**A**) Schematic illustrating concentration calculations. By
507   adding agonist solution of concentration c_y and volume V_y to a well with volume V_x and agonist
508   concentration c_x, the volume increases to V_{x+y} and the concentration increases to c_{x+y}. (**B**) Two
509   different modes for serial agonist addition. Mode 1: The total volume in the well V_{x+y} increases
510   with each addition. Mode 2: The volume added at each concentration step is removed again
511   before the next dose is added, which keeps the total volume in the well constant.
512
513   **Figure 2: Results of a typical experiment in 8-well format. (A)** Time course of impedance change
514   at 12 kHz for confluent U-373 MG cell layers grown on circular working electrodes of 250 µm
515   diameter. In seven of eight wells serial addition of histamine was applied. One well (grey curve)
516   was sequentially loaded with histamine-free buffer at each step (mode 1), except for the last step
517   when 100 µM histamine were added as a positive control. (**B**) Dose-response relationships
518   generated from the maximum impedance change relative to the baseline for seven individual
519   wells after each concentration step. (**C**) Average time course of impedance change (mean ± SD)
520   generated from seven individual wells as shown in (**A**). (**D**) Average dose-response relationships
521   generated from individual data sets as shown in (**B**).
522
523   **Figure 3: Adaptation of data analysis mode**s **(A)** Typical time course of impedance change at
524   12 kHz for a single confluent U-373 MG cell layer grown on circular working electrodes of 250 µm

diameter. **(B)** Experimental dose-response relationship (filled symbols) generated from the maximum impedance change relative to baseline after each concentration increase. Either the full data set was subjected to the fitting procedure (black and grey curve) or the last data point - indicating the onset of receptor desensitization and/or internalization - was omitted from quantitative analysis (red and magenta curve). All four parameters were adjusted during least square optimization (black and red curve) or the parameter A2, representing the lower asymptote, was set and fixed to zero (grey and magenta curve). EC_{50} values were not significantly different for the two data analysis modes.

Figure 4: Typical experiment in 96-well format. (A) Time course of impedance change measured at 12 kHz for confluent U-373 MG cell layers grown on a 96-well electrode array. Data are shown for 32 wells. 24 wells were subjected to a series of increasing histamine concentrations added with an equilibration time of 15 min between each step. The curve highlighted in red was excluded from averaging. Eight wells (control) were treated with histamine-free buffer at each time point of addition. **(B)** Time course of average impedance change for 23 individual wells in response to histamine serial addition and eight control wells as shown in **(A)**. **(C)** Average dose-response relationship generated from the maximum impedance change relative to the baseline after each concentration increase (mean ± SD). Data were fitted with a four parameter logistic function.

Figure 5: Typical experiment in antagonist mode. (A) Time course of impedance change at 12 kHz for confluent U-373 MG cell layers grown on circular working electrodes of 250 μm diameter upon addition of increasing concentrations of histamine with or without presence of histamine receptor antagonist diphenhydramine. Diphenhydramine (0.75 μM) or antagonist-free medium (L15) were added 20 min before the agonist serial dosing started. **(B)** Dose-response relationships generated from the maximum impedance change relative to the baseline after each concentration increase from data shown in (A). Presence of 0.75 μM Diphenhydramine increased the EC_{50} from (0.92 ± 0.05) μM to (14.7 ± 0.6) μM.

Table 1: Concentration calculation scheme for experiments using 8-well electrode arrays following addition mode 1.

Table 2: Pipetting scheme for experiments using 8-well electrode arrays following addition mode 1.

Table 3: Concentration calculation scheme for experiments using 8-well electrode arrays following addition mode 2.

Table 4: Pipetting scheme for experiments using 8-well electrode arrays following addition mode 2.

Table 5: Results obtained from four parameter logistic fits applied to typical dose-response data. The dose-response data used for analysis is presented in **Figure 2**. The full concentration range was applied to analysis for the individual wells one through seven (**Figure 2B**) and for the

569   average data obtained from wells one to seven (**Figure 2D**). None of the parameters of the logistic
570   function was fixed during least square optimization. Fit results were rounded to the decade of
571   the error, except if the value was smaller than the error.
572
573   **DISCUSSION:**
574   This protocol describes a method for label-free impedance measurements to determine the dose-
575   response relationship of agonist-induced GPCR activation in absence or presence of specific
576   antagonists for the same receptor. The proof of concept of this method was presented in a recent
577   publication[12]. To our knowledge it is the first study describing the establishment of a full dose-
578   response curve of agonist-mediated GPCR activation using a single cell layer in vitro. The
579   approach inevitably requires the use of label-free cell monitoring approaches and will not be
580   applicable to endpoint assays.
581
582   The protocol has been demonstrated on the example of U-373 MG cells, which endogenously
583   express the human histamine 1 receptor (hHR1) that is stimulated with a series of increasing
584   concentrations of its endogenous agonist histamine while the impedance of the cells is
585   continuously recorded. To demonstrate the applicability of the protocol to antagonist studies,
586   the experiments were repeated in presence of one constant concentration of diphenhydramine,
587   a competitive antagonist to the hH1R. Impedance recordings have also been used successfully to
588   study other H1R agonists (e.g., UR-KUM530[11], individual and stepwise dosing) and antagonists
589   (e.g., Mepyramine)[12] in addition to the examples shown here. Moreover, the protocol has been
590   applied to other cell lines and receptors as well, e.g. CHO-D2R expressing the dopamine D2
591   receptor (D2R) or BAEC expressing the ß$_2$-adrenergic receptor (ß$_2$AR)[12] indicating that it presents
592   a general scheme for very efficient generation of dose-response data. While U373-MG and BAEC
593   cells express the respective receptors H1R and ß$_2$AR at endogenous levels, the CHO-D2R is an
594   example for a recombinant cell model. Taken together, the serial protocol is in general applicable
595   to cell types with endogenous or ectopic GPCR expression, GPCRs with different coupling types
596   G$_q$ (e.g., H1R), G$_s$ (e.g., ß$_2$AR), G$_i$ (e.g., D2R) as well as different ligand types (agonist/antagonist).
597   In all those cell/receptor types mentioned above, the impedance increases with increasing
598   agonist concentration. However, some cells respond to GPCR activation by an impedance
599   decrease. We tested the protocol for one such case (HaCaT/histamine) and found also a
600   concentration-dependent stepwise decrease, supporting the notion of a generally applicable
601   approach that is also compatible with inverse impedance changes. It has been proposed that the
602   detailed time course of impedance data indicates the type of G-protein coupling of a given
603   receptor. Even though the concept is attractive, the pool of our data does not support a generally
604   applicable rule that correlates the impedance time profile to G-protein coupling of a given GPCR[6].
605
606   The serial addition protocol is adaptable to different types of electrode layouts and multi-well
607   formats. It is applicable to on-chip perfusion systems, which is a significant advantage over many
608   label-based approaches that often require one cell layer for one concentration to be tested.
609   Accordingly, the serial dosing scheme may pave the way to dose-response studies in more
610   complex biological models like organ-on-a chip or even human-on-a-chip approaches. Impedance
611   measurements detect an integrated, distal response of the cells to receptor activation that
612   ultimately induces cell morphology changes. This rather general and unspecific but also unbiased

613   readout is the basis for its broad applicability that is not restricted to GPCRs but might also work
614   for other classes of cell surface receptors, cytoplasmic receptors or even intracellular proteins as
615   targets.
616
617   It is critical for the stepwise agonist addition protocol to work with confluent cell layers on the
618   electrodes, as sparse cultures will interfere with sensitivity, reproducibility and data
619   interpretation. An additional prerequisite for successful monitoring of receptor activation even
620   in confluent cell monolayers is a change in cell shape along the signal transduction cascade. If the
621   cells under study do not change their morphology along the experiment, impedance-based
622   readouts are blind and will not report on any change in receptor activity. To properly layout the
623   serial addition protocol, initial pre-testing in conventional *one-well-one-concentration* mode is
624   recommended to determine the concentration range of the agonist roughly and the time interval
625   between subsequent agonist additions. Time intervals between sequential doses of the agonist
626   should be tailored such that the system adopts a new equilibrium before the next, higher dose is
627   added. Accordingly, the method may be less useful for receptors that trigger a very quick and
628   only transient response of the cells or a very slow response that may take hours to complete. The
629   latter situation would increase the total time of the experiment and call for parallel addition
630   protocols to cut short on lab time. In the examples presented above, the total runtime of the
631   experiment was 2.5 – 3.5 h, depending on the number of concentrations (8 or 10) and a potential
632   pre-incubation with antagonist. The same experiment in conventional parallel agonist addition
633   mode would only take ~ 1 h for the same cell-receptor model but with much smaller throughput.
634   A clear advantage of the serial approach is that the number of concentrations that need to be
635   tested for a full dose-response relationship is not limited by the number of available wells. If
636   higher agonist concentrations are needed, the experiment is easily extended and completed
637   without any additional cell culture work. Agonist addition itself should be carried out carefully,
638   as some cells are sensitive to shear stress and respond with considerable impedance changes
639   simply due to mechanical stimulation imposed by liquid handling. The cells might even come off
640   the electrode. This problem is, however, not unique to impedance-based cell monitoring but
641   applies to other techniques as well. For sensitive cells addition mode 1 is recommended due to
642   reduced mechanical load on the cells. However, this mode operates with lower liquid volumes
643   during addition which raises the risk of incomplete mixing since intensive agitation is not
644   recommended to avoid unspecific cell responses.
645
646   Addition mode 1 goes along with a stepwise increase of the total volume in the well whereas
647   mode 2 keeps the total volume constant since equal amounts of solution are sequentially
648   removed. These are just the two extreme strategies and might be adapted for special cell types,
649   multi-well formats or electrode layouts in any intermediate way. In the case of the U373-MG/H1R
650   model slight differences in EC_{50} and E_{Max} values have been observed for the serial addition mode
651   compared to the conventional parallel addition mode (serial, N = 30: EC_{50}: (0.8 ± 0.3) µM, E_{Max}:
652   (1.9 ± 0.2) kΩ; parallel, N = 15: EC_{50}: (0.5 ± 0.2) µM, E_{Max}: (1.3 ± 0.3) kΩ), while other cell/receptor
653   models did not show any differences. Shifts in EC_{50} and E_{Max} values could originate from receptor
654   desensitization that is more likely to occur when the receptors are exposed to the agonist for
655   longer times. The desensitization influence will strongly depend on the receptor and the ligand
656   under study. It remains to be elucidated whether a detailed analysis of serial and parallel agonist

657 addition may provide a new means to study receptor desensitization.
658

659 We recommend performing a control experiment with conventional parallel mode agonist
660 addition in order to test for potential shifts in dose-response curves. Further controls should
661 include a sample treated only with medium/solvent in appropriate doses to unravel any effects
662 due to solvent load or mechanical stress upon medium addition. This becomes particularly
663 important if the stock solution of the ligand is prepared in other solvents than medium (e.g.,
664 DMSO, ethanol). When investigating the effect of different ligands a standard agonist should be
665 included as reference.
666

667 Future directions should involve automatic liquid handling units, which might allow for
668 completely automated dosing or titration. Applying the serial addition mode in perfusion systems
669 holds great potential for lab-on-a chip and organ-on a-chip approaches, as well as for experiments
670 performed under liquid shear stress.
671

679 **DISCLOSURES:**
680 The authors have nothing to disclose.
681

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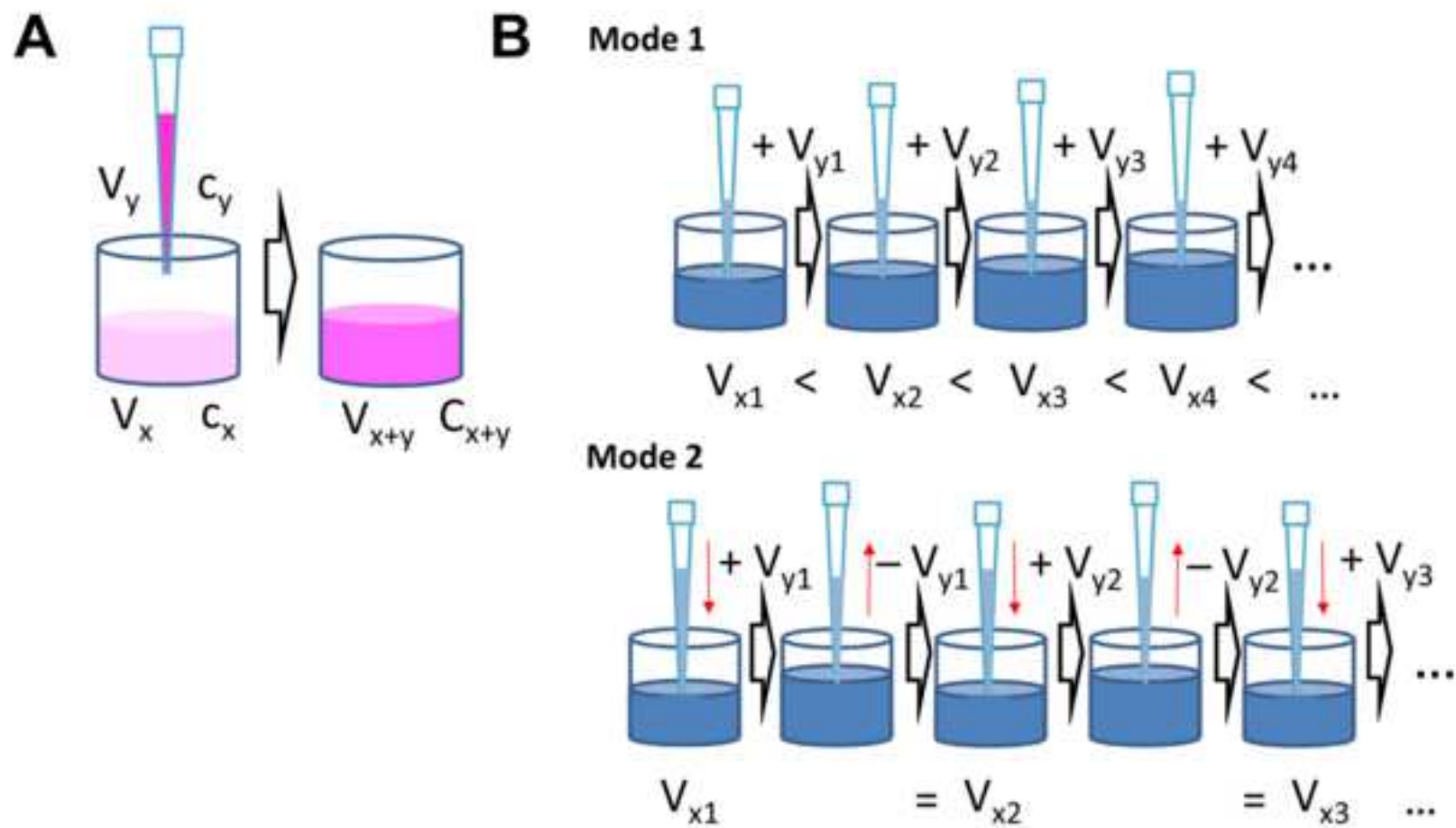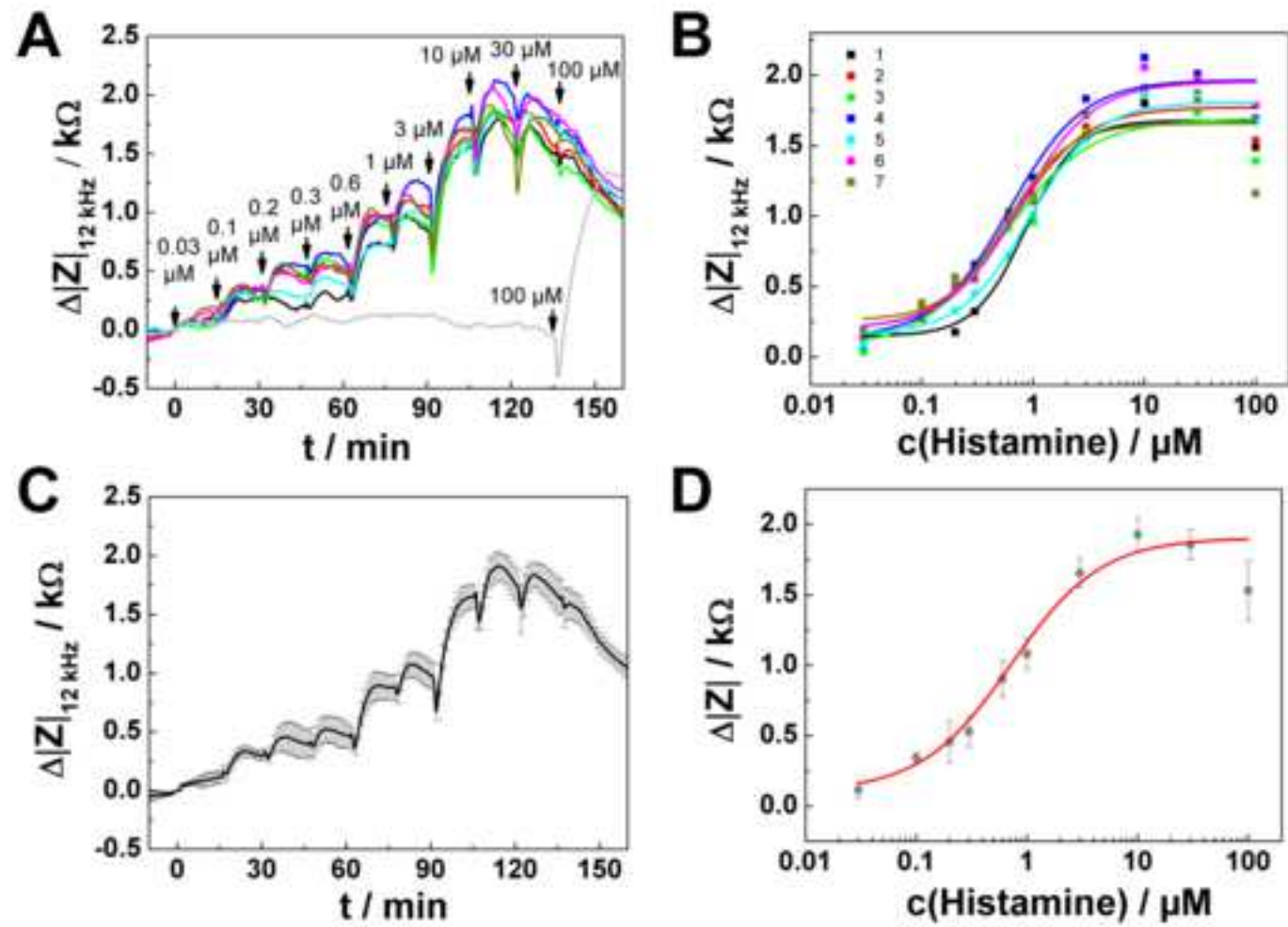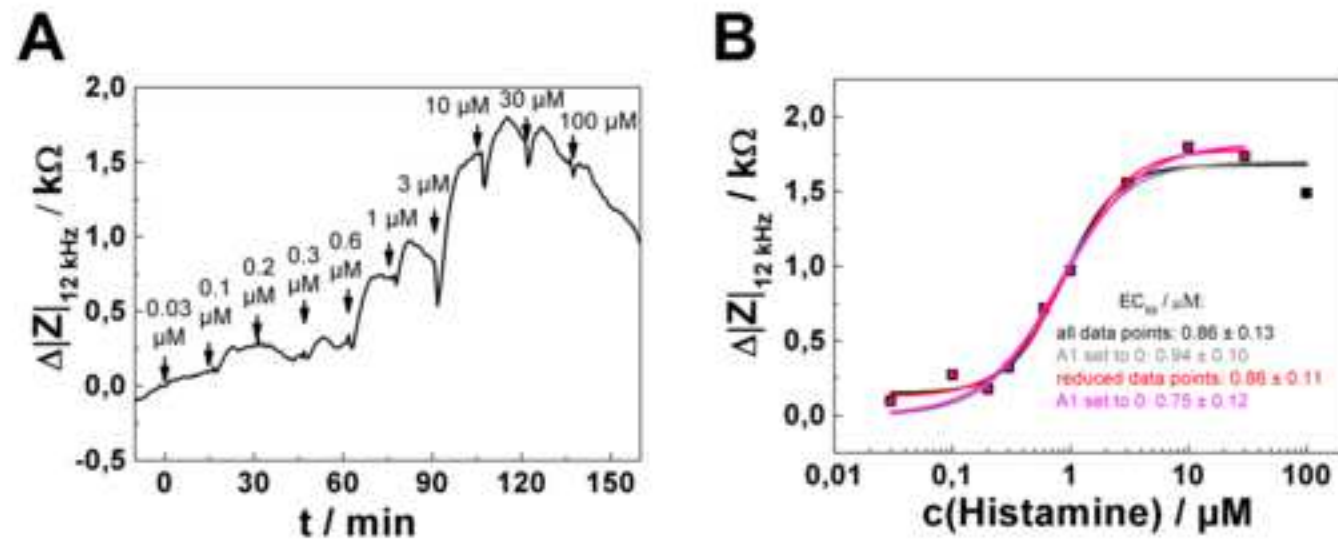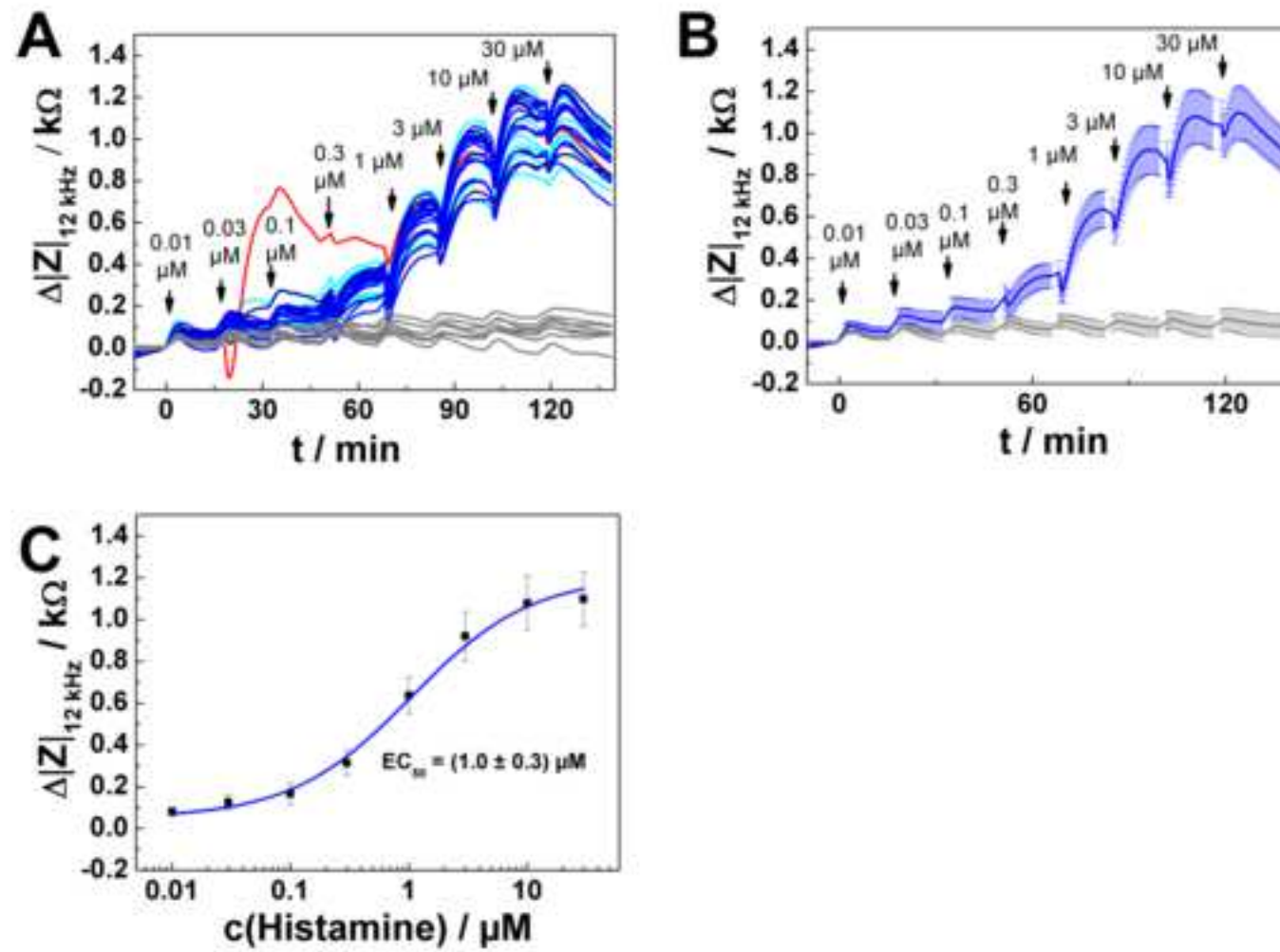712 *Biology* **11**, 10 (2019).
713

Figure 1

Figure 1

Figure 4

Figure 5

Table 1
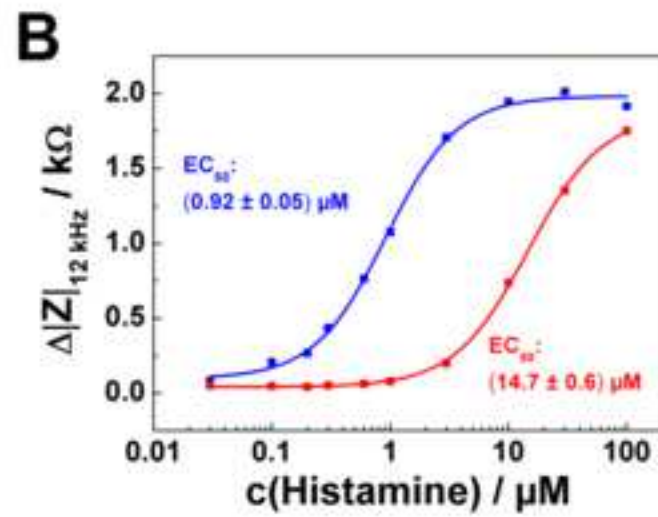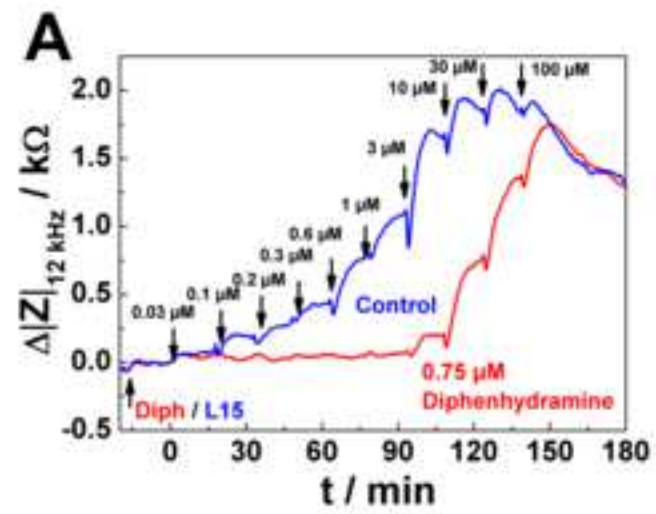
Solution #	c_{x+y} (µM)	c_x (µM)	V_x (µL)	V_{x+y} (µL)	V_y (µL)	c_y (µM)
1	0.01	0	200	230	30	0.08
2	0.1	0.01	230	260	30	0.79
3	0.3	0.1	260	290	30	2.03
4	1	0.3	290	320	30	7.77
5	3	1	320	350	30	24.33
6	10	3	350	380	30	91.67
7	30	10	380	410	30	283.33
8	100	30	410	440	30	1056.67

Solution #	c_{x+y} (µM)	c_x (µM)	V_x (µL)	V_{x+y} (µL)	V_y (µL)	c_y (µM)
1	0.01	0	200	230	30	0.08

Table 2

Solution #	c_y (μM)	V_y (μL)	make from Solution #	c (μM)	dilution factor	make total V (μl)	use (μl)	add V (μl)
1	0.08	30	3	2.03	26.52	600	22.6	577.4
2	0.79	30	4	7.77	9.83	600	61	539
3	2.03	30	5	24.33	11.97	600	50.1	549.9
4	7.77	30	6	91.67	11.8	600	50.8	549.2
5	24.33	30	7	283.33	11.64	600	51.5	548.5
6	91.67	30	8	1056.67	11.53	600	52.1	547.9
7	283.33	30	8	1056.67	3.73	600	160.9	439.1
8	1056.67	30	10 mM Stock	10000	9.46	800	84.5	715.5

Table 3

Solution #	c_{x+y} (µM)	c_x (µM)	V_x (µL)	V_{x+y} (µL)	V_y (µL)	c_y (µM)
1	0.01	0	200	400	200	0.02
2	0.1	0.01	200	400	200	0.19
3	0.3	0.1	200	400	200	0.5
4	1	0.3	200	400	200	1.7
5	3	1	200	400	200	5
6	10	3	200	400	200	17
7	30	10	200	400	200	50
8	100	30	200	400	200	170

Table 4

Solution #	c_y (µM)	V_y (µL)	make from Solution #	c (µM)	dilution factor	make total V (µl)	use (µl)	add V (µl)
1	0.02	200	3	0.5	25	1000	40	960
2	0.19	200	4	1.7	8.95	1000	111.8	888.2
3	0.5	200	5	5	10	1000	100	900
4	1.7	200	6	17	10	1000	100	900
5	5	200	7	50	10	1000	100	900
6	17	200	8	170	10	1000	100	900
7	50	200	8	170	3.4	1000	294.1	705.9
8	170	200	200 µM Stock	200	1.18	1300	1105	195

Table 5

Well	EC_{50} (μM)	E_{max} (=A_2) (kΩ)	A_1 (Ω)	n
1	0.9 ± 0.1	1680 ± 70	150 ± 80	1.9 ± 0.5
2	0.7 ± 0.2	1770 ± 90	200 ±140	1.3 ± 0.4
3	0.6 ± 0.2	1700 ± 100	60 ± 250	1.1 ± 0.5
4	0.6 ± 0.2	2000 ± 100	140 ± 180	1.3 ± 0.4
5	0.9 ± 0.1	1810 ± 60	160 ± 80	1.4 ± 0.3
6	0.8 ± 0.1	1950 ± 70	200 ± 110	1.3 ± 0.3
7	0.6 ± 0.3	1700 ± 200	260 ± 250	1.6 ± 1.0
1 − 7	0.7 ± 0.2	1900 ± 100	100 ± 100	1.1 ± 0.2

Table of Materials

Name of Material/Equipment	Company	Catalog Number
Bürker counting chamber	Marienfeld (Lauda-Königshofen, Germany)	640210
cell culture flasks 25 cm^2	Greiner bio-one (Frickenhausen, Germany)	690175
Cell incubator (Heraeus Function Line BB15)	Thermo Scientific (Darmstadt, Germany)	\
Centrifuge (Heraeus 1S-R)	Thermo Scientific (Darmstadt, Germany)	\
Diphenhydramine hydrochloride	Sigma Aldrich (Taufkirchen, Germany)	D3630
Eagle's Minimum Essential Medium with 4.5 g/L D-Glucose and 2.2 g/L NaHCO$_3$	Sigma Aldrich (Taufkirchen, Germany)	D5671
Impedance Instrument (ECIS Zθ)	Applied BioPhysics Inc. (Troy, NY, USA)	\
8-well electrode Arrays (8W1E PET)	Applied BioPhysics Inc. (Troy, NY, USA)	\
96-well electrode arrays (96W1E+ PET)	Applied BioPhysics Inc. (Troy, NY, USA)	\
Fetal calf serum (FCS)	Biochrom (Berlin, Germany)	S0615
Histamine dihydrochloride	Carl Roth (Karlsruhe, Germany)	4017.1
Laminar flow hood (Herasafe, KS 12)	Thermo Scientific (Darmstadt, Germany)	51022515
Leibovitz' L-15 medium	Thermo Scientific (Darmstadt, Germany)	21083-027
L-glutamine	Sigma Aldrich (Taufkirchen, Germany)	G7513
Micropipette large (100 - 1000 µL)	Brandt (Wertheim, Germany)	704780
Micropipette large (20 - 200 µL)	Brandt (Wertheim, Germany)	704778
Microscope (phase contrast, Nikon Diaphot)	Nikon (Düsseldorf, Germany)	
Penicillin/streptomycin	Sigma Aldrich (Taufkirchen, Germany)	P0781
Phosphate buffered saline (PBS)	Sigma Aldrich (Taufkirchen, Germany)	D8537
Pipette, serological	Greiner bio-one (Frickenhausen, Germany)	607 180
Pipettor (accu-jet pro)	Brandt (Wertheim, Germany)	26300
Trypsin	Sigma Aldrich (Taufkirchen, Germany)	T4174
Tube, 15 mL	Greiner bio-one (Frickenhausen, Germany)	188 271

Tube, 50 mL	Greiner bio-one (Frickenhausen, Germany)	210 261
U-373 MG cells	ATCC (Rockville, MD, USA)	ATCC HTB-17
water bath (TW21)	Julabo (Seelbach, Germany)	\

Comments/Description

PET base with 0.049 mm² working electrode and ~50 mm² counter electrode (gold)
PET base with two electrodes (gold) with 0.256 mm² total electrode area

class II safety cabinet

 in PBS with 1 mM EDTA

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Editor
Journal of Visual Experiments

Unser Zeichen (Bitte bei Antwort angeben)	Ihr Zeichen / Ihre Nachricht vom	Ihr Ansprechpartner + 49 941 943-4546	Regensburg, den 20.10.2019

Submission of Revised Manuscript R1

Dear Editor,

our manuscript entitled "Stepwise dosing protocol for increased throughput in label-free impedance-based GPCR assays" has been revised a second time for the issues mentioned in your email. Please find below a point-by-point response to the various issues. We hope that our study is now acceptable for the "Journal of Visual Experiments".

On behalf of all co-authors, yours sincerely

Joachim Wegener

Editor / Reviewer Comments blue
Authors' Response black

Editorial comments:
The manuscript has been modified and the updated manuscript, **60686_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
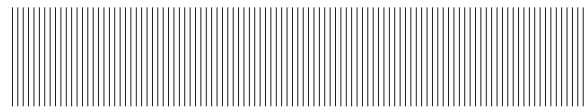
We have checked and revised the manuscript for spelling and grammar issues using the software-based spell checker and careful reading. To the best of our language skills the text should now be appropriate.

We have boiled down all *notes* down to no more than 4 lines except for one single case, in which we describe the calculation of the concentration scheme. Here, we consider it essential to be more detailed. Putting it some other place would make the manuscript less readable and instructive.

The numbering has been corrected. However, we did not find a problem with step 3.3.4 (there is no step 3.3.4) but we corrected the numbering of steps 6.3.5 through 6.3.11 in the revised manuscript.

The numbering has been corrected. However, we did not find a problem with step 3.3.7 (there is no step 3.3.7) but we corrected the numbering of steps 6.3.5 through 6.3.11 in the revised manuscript.

The numbering has been corrected. However, we did not find a problem with step 3.3.8 (there is no step 3.3.8) but we corrected the numbering of steps 6.3.5 through 6.3.11. in the revised manuscript.