Video Article

A Chemical Screening Procedure for Glucocorticoid Signaling with a Zebrafish Larva Luciferase Reporter System

Benjamin D. Weger[1], Meltem Weger[1], Nicole Jung[2], Christin Lederer[1], Stefan Bräse[1,3], Thomas Dickmeis[1]

[1]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology - Campus North

[2]Institute of Organic Chemistry, Karlsruhe Institute of Technology - Campus North

[3]Institute of Organic Chemistry, Karlsruhe Institute of Technology - Campus South

Correspondence to: Thomas Dickmeis at thomas.dickmeis@kit.edu

URL: http://www.jove.com/video/50439
DOI: doi:10.3791/50439

Keywords: Developmental Biology, Issue 79, Biochemistry, Vertebrates, Zebrafish, environmental effects (biological and animal), genetics (animal), life sciences, animal biology, animal models, biochemistry, bioengineering (general), Hormones, Hormone Substitutes, and Hormone Antagonists, zebrafish, *Danio rerio*, chemical screening, luciferase, glucocorticoid, stress, high-throughput screening, receiver operating characteristic curve, *in vivo*, animal model

Date Published: 9/10/2013

Citation: Weger, B.D., Weger, M., Jung, N., Lederer, C., Bräse, S., Dickmeis, T. A Chemical Screening Procedure for Glucocorticoid Signaling with a Zebrafish Larva Luciferase Reporter System. *J. Vis. Exp.* (79), e50439, doi:10.3791/50439 (2013).

Abstract

Glucocorticoid stress hormones and their artificial derivatives are widely used drugs to treat inflammation, but long-term treatment with glucocorticoids can lead to severe side effects. Test systems are needed to search for novel compounds influencing glucocorticoid signaling *in vivo* or to determine unwanted effects of compounds on the glucocorticoid signaling pathway. We have established a transgenic zebrafish assay which allows the measurement of glucocorticoid signaling activity *in vivo* and in real-time, the GRIZLY assay (Glucocorticoid Responsive *In vivo* Zebrafish Luciferase activitY). The luciferase-based assay detects effects on glucocorticoid signaling with high sensitivity and specificity, including effects by compounds that require metabolization or affect endogenous glucocorticoid production. We present here a detailed protocol for conducting chemical screens with this assay. We describe data acquisition, normalization, and analysis, placing a focus on quality control and data visualization. The assay provides a simple, time-resolved, and quantitative readout. It can be operated as a stand-alone platform, but is also easily integrated into high-throughput screening workflows. It furthermore allows for many applications beyond chemical screening, such as environmental monitoring of endocrine disruptors or stress research.

Video Link

The video component of this article can be found at http://www.jove.com/video/50439/

Introduction

Glucocorticoids (GCs) are steroid hormones produced by the adrenal gland which play important roles during the stress response and in the regulation of metabolism[1]. GCs bind cytoplasmic glucocorticoid receptors (GRs) of the nuclear receptor superfamily which, upon binding, translocate into the nucleus[2]. Here, they can for example repress transcription by interfering with other transcription factors (transrepression) or activate gene transcription via glucocorticoid response elements (GREs, transactivation). Due to their anti-inflammatory properties, both natural and artificial GCs are widely used drugs in the treatment of a number of diseases, such as asthma or arthritis[3]. However, especially long-term use of GCs can lead to severe side effects, including diabetes and glaucoma[4]. Therefore, novel compounds targeting GC signaling with potentially more beneficial treatment efficiency and tolerability are highly sought after. Importantly, ligand effects observed in cultured cells can differ from those seen *in vivo*[5]. Such effects might bias results obtained with conventional cell culture based pharmaceutical screening assays. Chemical *in vivo* screening as enabled by the zebrafish model has recently come into focus, as it allows the determination of effects not detectable in cell culture[6,7].

Hormonal signaling pathways can also be affected by environmental pollutants. So-called endocrine disrupting chemicals (EDCs) affect various hormone regulated processes[8]. Thus, reproduction and sexual differentiation of aquatic organisms can be modulated by substances with estrogen-like activities. Recently, concerns have been raised that metabolic disorders might be linked to EDCs in the environment[9]. One pathway targeted by such "metabolic disruption" is the glucocorticoid pathway, which has also been implicated in xenobiotics effects on development and immune function[10,11]. However, compared with the large amount of information available on compounds interfering with sex steroid hormone action, relatively little is known about endocrine disruption effects mediated via the GR. Therefore, tools are needed that allow monitoring pollutant effects on GC signaling *in vivo*.

The zebrafish has long been a popular model in developmental biology and has more recently also attracted researchers from other fields, including endocrinology[12]. Compared with other teleosts, the GC signaling system of zebrafish is more similar to that of mammals, since the zebrafish genome contains only one GR gene as opposed to the duplicated receptors in many other fish species[13-15]. In addition, the

hypothalamic-pituitary-adrenal axis is already functional in 5 day old zebrafish larvae, which increase endogenous GC production in response to stressors[14,16-18].

We have recently generated a transgenic zebrafish line, GRE:Luc, which allows for the monitoring of GC signaling activity *in vivo* and in real-time[18]. The line carries a luciferase reporter gene construct under control of a minimal TATA box promoter and four concatemerized GREs (**Figure 1a**). GC induced bioluminescence can be measured from single GRE:Luc larvae in 96 well microtiter plates *in vivo* over prolonged periods of time. This GRIZLY assay (for "Glucocorticoid Responsive *In vivo* Zebrafish Luciferase activitY") can be used in a number of different research fields, such as stress research, environmental monitoring, and pharmacological screens[18]. We were able to detect the endogenous rise in cortisol after osmotic stress from single larvae and could follow the maturation of the response during development. Furthermore, we could monitor the effects on GC signaling of organotins that require metabolization by the larva. Importantly, the line was able to detect these effects at environmentally relevant concentrations. Finally, in a pilot screen the assay sensitively and specifically detected compounds with GC activity from a chemical library, including one compound that stimulated endogenous cortisol production in the larvae. Here, we describe a detailed protocol for chemical screens using the GRIZLY assay.

Protocol

1. Prepare Working Dilution of Chemical Library

Predilute the compounds of the drug library to be tested (*e.g.* FDA approved drugs, ENZO Life Science) into E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$) with a robotic liquid handling station (*e.g.* Multiprobe II, 8 needles with disposal tip adapter, PerkinElmer). Suitable concentrations are *e.g.* 40 µg/ml in 3% DMSO, but this will depend on the type of library used. The Multiprobe II settings corresponding to the steps described below are indicated in **Table 1**. The protocol described in the table allows the preparation of four aliquot plates in parallel.

1. Pipette 10 µl aliquots of the stock library (2 mg/ml in DMSO) into deep well plates (*e.g.* 96-Well Storage Plate, Round Well, 0.8 ml, ABgene). Columns 1 and 12 should be left empty - these will receive the positive and negative within-plate controls.
2. Dispense 490 µl of E3 with 1% DMSO into the prepared aliquots of the library with the Multiprobe II. Dilution is performed with fixed needles without disposal tips.
3. Add 10 µl of DMSO into column 1 and 10 µl of a 5 mM dexamethasone solution (in DMSO) to column 12 as within-plate controls. The concentration of DMSO in all wells is now 3%, the dexamethasone concentration in the positive control wells is 100 µM in E3/3% DMSO.
4. Seal the plate with DMSO resistant adhesive sealing sheets. Store the plates at -80 °C until usage.

2. Breeding of Reporter Fish

Collect embryos from natural spawning of group matings between GRE:Luc transgenic fish. Raise embryos (not more than 60) in 9 cm Petri dishes containing E3 medium supplemented with 1 mg/ml of the fungicide methylene blue until 4 days post fertilization (dpf) in an incubator at 28 °C. Change E3 medium regularly.

3. Preparation of Luciferase Medium (E3L)

Prepare an aqueous luciferin stock solution of 50 mM by adding dH_2O to the vial containing the luciferin powder. This stock solution can be stored at -80 °C for several months. Dilute the luciferin stock into E3 medium to a final concentration of 0.5 mM to obtain E3L medium.

4. Distribute Larvae into 96 Well Plates

1. Prepare 1 ml pipette tips with wide bore by cutting off approximately 5 mm of the tip and briefly flaming the sharp edges.
2. Pool larvae from several crosses by carefully pouring them from the Petri dishes into a beaker. Harvest about 50 larvae at a time by gently pouring them onto a sieve (pore size of 0.25 mm diameter) and immediately place the sieve into a small Petri dish (diameter 5.5 cm) filled with E3L medium.
3. With a wide bore pipette tip, transfer 225 µl of medium containing one larva each to the wells of a white 96 well plate (OptiPlate, PerkinElmer).
4. Seal the plates with adhesive sealing sheets (*e.g.* TopSeal-A, PerkinElmer) and incubate them at 28 °C overnight. This preincubation prevents recording of transient changes in bioluminescence immediately after addition of luciferin, a phenomenon that occurs also in cultured cells[19].

5. Drug Treatment

1. Remove the plate containing the working dilution of the library from the -80 °C freezer about 4 hr before use. Gently vortex the plate and briefly spin it in a centrifuge to collect the liquid at the bottom of the plate.
2. Remove adhesive sealing sheets from the larvae plates.
3. With a hand-operated 96-channel pipetting device (*e.g.* Liquidator, Steinbrenner) pipette the drug solution up and down 3 times. Then aliquot 25 µl of the working dilution of the chemical library into the wells containing the larvae (with the example above, this results in a final concentration of 4 µg/ml in E3 with 0.3% DMSO). Prepare 10 (minimum: 5) replica plates with larvae per library plate.
4. Seal the plates with the adhesive sealing sheets and label each plate with a barcode sticker.

6. Luminescence Recording

1. Put the plates containing the larvae into the stacking units of an EnVision bioluminescence reader with enhanced luminescence sensitivity (PerkinElmer). Alternatively, use a robotized incubator system such as those employed for mammalian cell culture screening systems in combination with the reader.
2. Record bioluminescence for two days using reader settings analogous to those described in **Table 2** for the EnVision reader. Adapt the number of assay repeats to the number of plates in the run to match the required running time.
3. After the end of the run, check the plates for the presence of dead larvae to evaluate general toxicity of the compounds.

7. Data Analysis

All data analysis is performed in the statistical programming environment R using custom scripts.

1. AUC calculation
   In order to identify compounds activating GC signaling regardless of their kinetic properties, determine the area under the curve (AUC) of the recorded luminescence traces (bioluminescence raw counts vs. time) as the screening parameter. AUCs are approximated with the trapezoidal rule (see **Figures 2a** and **a'**).
2. Normalization
   Log transform the AUC values in order to ensure a higher normality of the data (**Figures 2b** and **b'**). Then normalize the log transformed AUC values for each library plate with the robust *Z*-score method (see [20]). This normalization results in values representing the number of standard deviations from the median of all data points. In this way, systematic errors, such as inter-run variability, are removed from the data. The data can now be visualized by plotting the mean robust *Z*-score of the replicas for each well (**Figures 2c** and **c'**).
3. Quality metrics
   1. Heatmap
      In order to visualize plate related effects, plot the data for each plate as a heatmap using *e.g.* the *heatmap.2* function of the gplot package[21]. In this way, edge effects or compound carry-over can be easily detected (*e.g.* compare **Figure 3a** with **3b**). Exclude sub-optimal plates from further analysis.
   2. ROC curve
      To determine sensitivity and specificity of the assay, calculate a receiver operating characteristic (ROC) curve *e.g.* with the help of the ROC bioconductor package[22] using the *Z*-score values determined in 7.2 (**Figure 3c**). Subsequently, determine the AUC of this curve. An AUC value close to 1 indicates high sensitivity and specificity of the assay.
4. Hit Identification
   To optimize the cut-off value for the identification of active compounds, determine the Youden Index. To calculate the index, subtract the estimated true positive rate (TPR) from the false positive rate (FPR) of the ROC curve for increasing robust *Z*-score cut-off values. Take the robust *Z*-score with the highest Youden Index (TPR minus FPR) as the cut-off point. The TPR/FPR can be estimated by detection of positive control wells or of known active compounds within the library. When using positive control wells make sure that they match the anticipated hit strength.

8. Retesting

1. Reevaluate positive hit compounds by treating larvae with serial dilutions of the compounds obtained from a different supplier, using the same recording set-up described above. True hits should induce luminescence also in this assay, ideally in a dose dependent manner.

Representative Results

In a previous publication[18], we screened a library of 640 FDA approved drugs in a pilot screen to evaluate the performance of the GRIZLY assay. This library contained 12 *bona fide* GCs, thereby allowing us to determine quality measures for sensitivity and specificity of the assay.

Figure 2 shows typical examples of the raw data analysis and normalization taken from this screen. A typical result from a dexamethasone control well is depicted in **Figure 2a**, illustrating the temporal information given by the data. A peak in bioluminescence that occurs at about 12 hr after the treatment slowly decreases over the following 36 hr of measurement. **Figure 2a'** highlights the approximation of the AUC value using the trapezoidal method. This calculation is carried out for all wells and each technical repeat. The result of the log transformation is shown in **Figures 2b** and **b'**. Here the mean of the positive controls are plotted in a normal Q-Q plot. Log transformation leads to a greater approximation of the data points to the theoretical normally distributed values indicated by the red diagonals. A transformation to achieve normality of the data increases the statistical power of the subsequent analyses. Finally, **Figures 2c** and **2c'** show the effect of robust *Z*-score normalization on plate-to-plate variability: The different baseline values obtained with different plates (**Figure 2c**) are normalized in the robust *Z*-score plot in **Figure 2c'**.

The robust *Z*-score values can be used to identify systematic errors in the data by visualizing the distribution of the hits across the plates. **Figure 3a** shows one example of a heatmap of a library plate, in which the robust *Z*-score values are plotted color-coded into the well positions. One easily identifies column 12 with the in-plate positive controls. Positive scoring library compounds are seen in well F8 and, albeit weaker, E2. **Figure 3b** shows a plate in which a systematic error is present. One observes a series of compounds with decreasing *Z*-score values in rows A and E over several columns. None of the compounds in these wells was tested positive in the re-test. Since the wells with this decreasing GRIZLY test activity are placed along the path the pipetting robot takes when aliquoting the library plates, this pattern is indicative of carry-over of a positive compound during the automated pipetting process.

The robust *Z*-score normalization together with the presence of known GCs in the library also enabled us to calculate a receiver operating characteristic (ROC) curve for the screen[18]. **Figure 3c** shows a plot of the estimated true positive rate against the estimated false positive rate

for different robust *Z*-score cut-off values. The estimated true positive rate is defined as the percentage of true positive hits (here, the known glucocorticoids present in the library) that are found at a given robust *Z*-score cut-off value. The corresponding estimated false positive rate indicates the percentage of non-positive compounds hit at this cut-off value. The AUC of this curve is 0.95, indicating a high sensitivity and specificity of the screen and excellent assay performance. The AUC curve was also used to estimate the optimal cut-off value for hit identification by calculating the Youden index for different robust *Z*-scores. We identified the robust *Z*-score of 1.49 as having the highest Youden index. This cut-off value is indicated in **Figure 2c'** as a black line separating the hits from the bulk of the compounds.

In our screen[18], we were able to identify nearly all known GCs present in the library. Only three of the 12 *bona fide* GCs were not detected. In the case of melengestrol acetate, this was a false negative finding, since this compound tested positive in the re-test at a higher concentration than the one used in the library. The other two GCs were negative also in the re-test. Corticosterone is the major GC in rodents, but not in fish or humans[1], while the prodrug prednisone might not be metabolized well by the larval system. Interestingly, also two drugs not annotated as GCs were identified in the screen, of which one could not be confirmed in the re-test (spironolactone, a mineralocorticoid receptor antagonist). The other compound, pregnenolone, is a precursor in steroid biosynthesis, which is converted to cortisol by the adrenal gland. Indeed, treatment with pregnenolone stimulated cortisol production in the larvae[18]. All these results confirm the high performance of the assay: only one false negative and one false positive compound were among the primary screen results, and 10 of the 11 confirmed compounds with GC signaling stimulating activity were already identified in the primary screen.

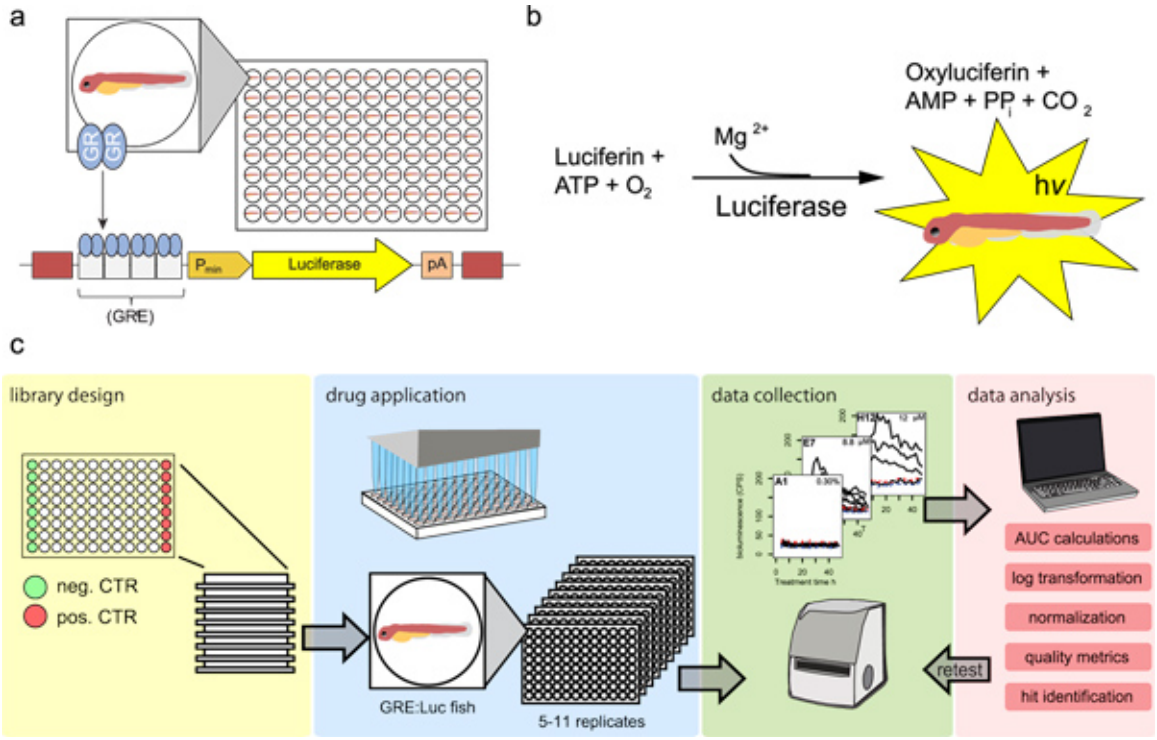

Figure 1. General principle of the assay and work flow of the screening procedure. a) Scheme of the GRIZLY assay reporter construct. Luciferase (yellow) expression is controlled by a minimal promoter (P_{min}, orange) and four concatemerized GRE elements ($(GRE)_4$, white), which are bound by ligand-activated GR homodimers (GR, blue). Red boxes indicate Tol2 transposase sites that facilitate integration of the construct into the genome. The pink box represents a poly-adenylation site (pA). Transgenic larvae carrying this construct are placed in opaque 96 well microtiter plates for bioluminescence measurements. **b)** Scheme of the luciferase reaction. Luciferase catalyzes the oxidation of luciferin to oxyluciferin, which leads to light emission (hv). The reaction requires also ATP and Mg^{2+}, which is provided by the larva. PP_i, pyrophosphate. **c)** Workflow of the screen. Working stock dilutions are prepared from an FDA approved drug library in 96 well plates, one column containing a negative within-plate control (DMSO only, green) and one column containing a positive control (Dexamethasone, red) (library design). GRE:Luc larvae are distributed in 96 well plates (5-11 replicates) and treated with the library compounds (drug application). Bioluminescence traces are recorded with a bioluminescence reader for two days (data collection). Bioluminescence traces are integrated (AUC calculations), log transformed and robust *Z*-score normalized (normalization). Normalized data are used for determining quality metrics and for hit identification (data analysis). Chosen hits are retested for dose-dependent activity in the assay (retest). Click here to view larger figure.

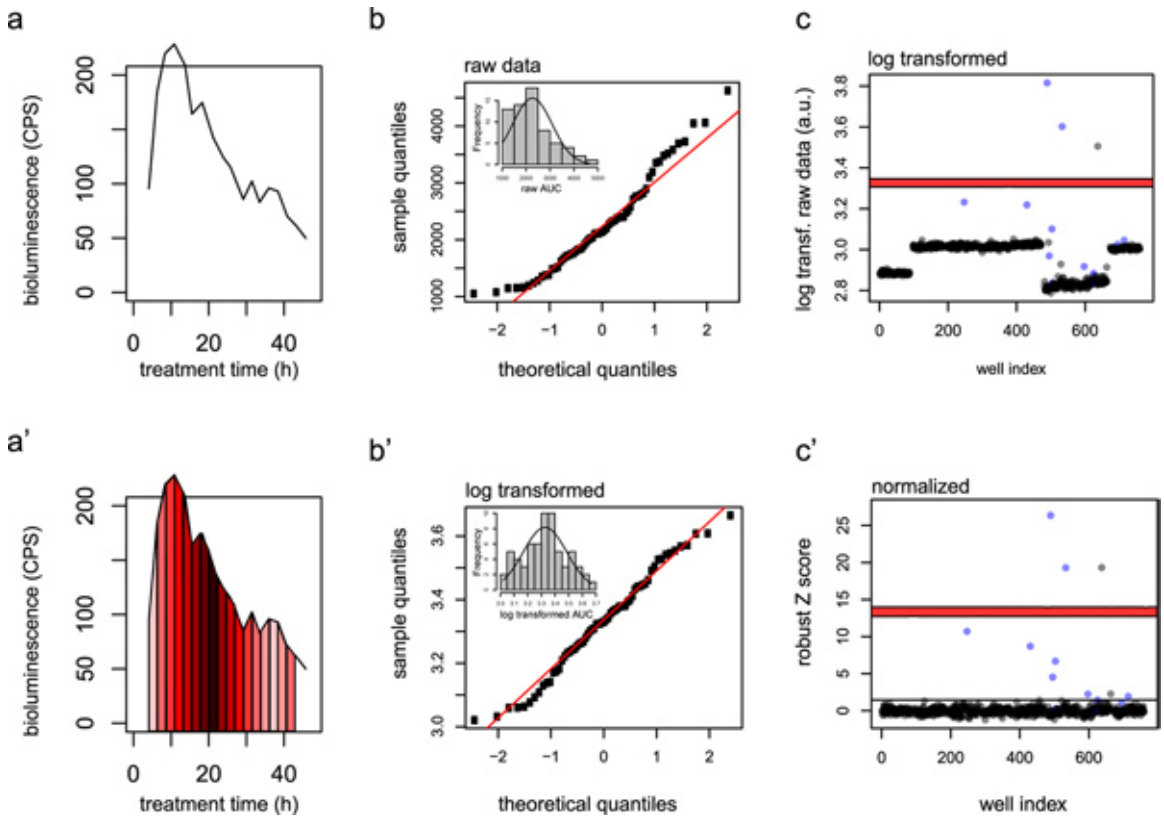

Figure 2. Data analysis illustrated with data from a screen of a FDA approved drug library. a) Representative trace from a positive control well. **a')** The area under the curve (AUC) is approximated with the trapezoid rule. The trapezoids used for the calculation are shown in different shades of red. **b)** Normal Q-Q plot of raw AUC values of the positive control wells (y-axis) vs. a standard normal population (x-axis) before log transformation. **b')** Normal Q-Q plot of the AUC values after log transformation. A normal distribution is indicated by the linearity of the log transformed data points. **c)** Plot of log transformed raw data for all compounds tested in the screen. Blue, *bona fide* glucocorticoids; grey, all other compounds. **c')** Plot of screen data after robust *Z*-score normalization. Systematic errors such as inter-plate variations have been removed from the data. Red lines indicate the mean ± s.e.m. of positive within-plate controls. Black line: hit identification cut-off value as determined by Youden-Index optimization (**Figure 3**). Reproduced with permission from[14], 2012 ACS. Click here to view larger figure.

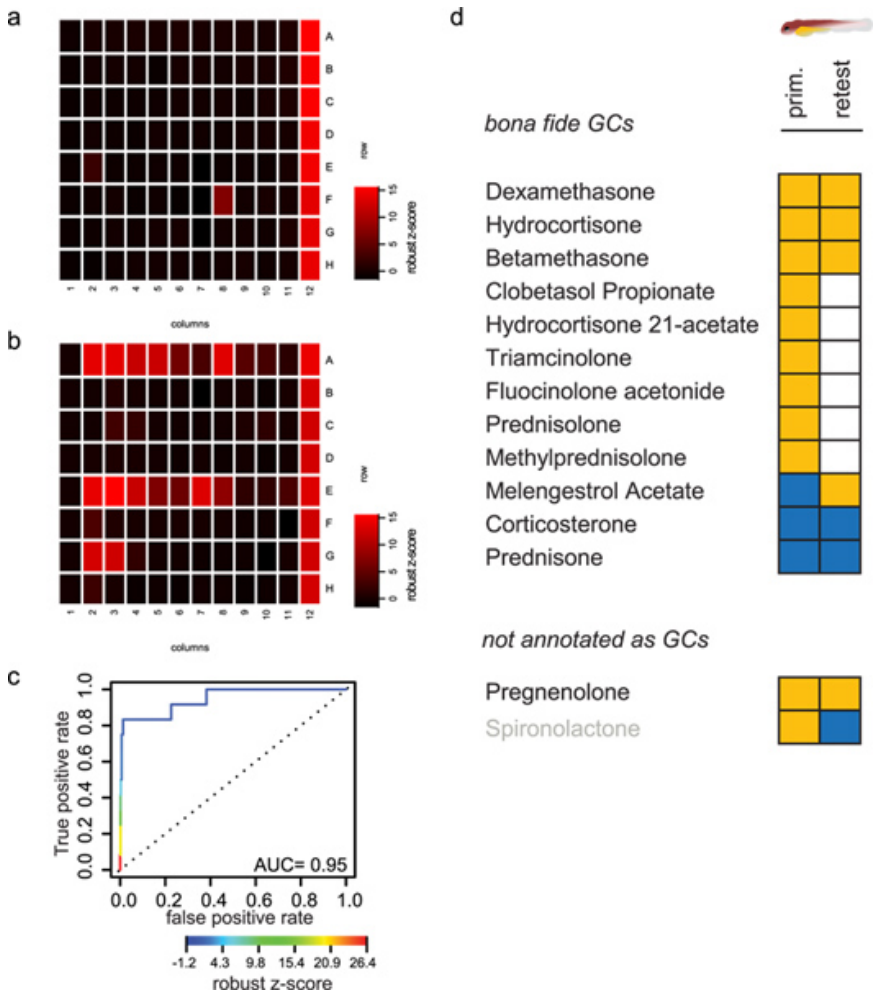

Figure 3. Screen results. a) Heatmap of screen results. Robust *Z*-score values of library compounds are plotted into the respective well positions. The positive controls in column 12 as well as two positive hit compounds in wells F8 and E2 are visible. **b)** Heatmap of a plate showing carry-over of a positive compound during library preparation with a pipetting robot. A gradient of activity is visible along the pipetting path taken by the robot. **c)** Receiver operating characteristic (ROC) curve for the screen. The estimated true positive rates (left-hand y-axis) and false positive rates (x-axis) are plotted for increasing robust *Z*-score cut-off values (color coded, right-hand y-axis). The AUC value of the resulting curve is close to 1, indicating good assay performance. Reproduced with permission from [14], 2012 ACS. **d)** Table showing compounds active (yellow) or inactive (blue) in the primary screen (prim.) or in the retest. *Bona fide* glucocorticoids were not always retested (white). Redrawn with permission from[14], 2012 ACS. Click here to view larger figure.

General settings:			
Step 1.1			
Procedure	type:	single liquid	
	mode:	blow out	
	dispenses per aspirate:	1	
	optimize for speed:	Yes	
	air gaps system:	10 µl	
	transport:	0	
Flush/Wash	**No**		
Aspirate	**Type of setting**	**Value**	**remarks**
	Position	B7, B10, B13, B16	
	Aspirate Vol.	10 µl	
	Speed	200 µl/sec	
	Liquid tracking	60%	
	Aspirate Height	Labware default	22.28
Dispense	**Type of setting**	**Value**	**remarks**
	Positions	D7, D10, D13, D16	
	Dispense Vol.	10 µl	
	Speed	200 µl/sec	
	Liquid tracking	100 µl	
	Dispense Height	Labware default	17.01
Step 1.2			
Procedure	**type:**	**reagent**	
	mode:	waste	
	dispenses per aspirate:	3	
	optimize for speed:	Yes	
	air gaps system:	15 µl	
	transport:	0	
Flush/Wash		**No**	
Aspirate	**Type of setting**	**Value**	**remarks**
	Position	A4	
	Aspirate Vol.	3 x 490 µl	
	Air gaps	15 and 0	
	Speed	200 µl/sec	
	Liquid tracking	400%	
	Aspirate Height	Liquid surface	
Dispense	**Type of setting**	**Value**	**remarks**
	Position	D7, D10, D13, D16	
	Dispense Vol.	490 µl	
	Air gaps	15 and 0	
	Speed	200 µl/sec	
	Liquid tracking	100%	
	Dispense Height	Labware default	17.01

Table 1. Settings for Multiprobe II. The protocol in **Table 1** describes the preparation of 4 plates (each 96 wells). The plates are located on plate adapters in the working area at positions specified by the robot (*e.g.* B7, B10, B13, B16).

General settings:			
Number of assay repeats		*dependent on the length of the run*	
Number of plates		unlimited	
Temperature control		28 °C	
Protocol		**Calculations**	
	US Lum 96 (cps) read		
	Measurement time	2.5 sec	Crosstalk correction
	Distance between plate and detector	0 mm	
	Glow (CT2) correction factor	0 %	

Table 2. EnVision settings used for the screen.

Discussion

We present here the workflow and data analysis for a chemical screen measuring GC activity *in vivo* using the GRIZLY assay[18]. The assay has quality control characteristics and performance measures that are comparable with conventional cell based screens, an important advantage for its use in high-throughput settings. In addition, however, the *in vivo* assay detects also compounds that are not accessible to *in vitro* screens. This is exemplified by the presence of the prohormone pregnenolone among the hits. Thus, the GRIZLY assay extends the scope of screens aimed at GC signaling activity.

The importance of the detection of *in vivo* effects with our assay is also illustrated by results we obtained with the organotin pollutants DBT and TBT[18]. DBT, but not TBT, has been shown to inhibit GC signaling in mammalian cell culture, and we observed the same in zebrafish cell cultures expressing the GRE:Luc reporter. Importantly however, in the GRIZLY assay, TBT showed inhibitory activity on the GC pathway, as it can be converted to DBT by the larval metabolism. This inhibition was observed already at environmentally relevant concentrations of TBT. These results further illustrate the potential of the GRIZLY assay in detecting compound effects based on inter-organ cross-talk and metabolic modifications of the compounds in the living animal. They also highlight the application potential of the GRIZLY assay for environmental monitoring. Thus, endocrine disruptor effects can be studied at the level of receptor signaling, where the disruptor interferes with GC signaling activity induced by a GR agonist such as dexamethasone. Another possibility is to study compound effects on pregnenolone-stimulated GC synthesis. The high throughput quality of the assay should permit the rapid screening of environmental sample libraries.

The use of luciferase as a reporter gene allows for a high dynamic range of detection of signaling activity[23]. Indeed, the sensitivity of the assay makes it possible to detect osmotic stress-induced GC production from single larvae[18]. The high temporal resolution achieved with the luciferase reporter allows us to follow signaling activities over time, which makes it possible to analyze also the kinetics of the data.

A potential drawback for some applications is the limited suitability for spatial monitoring of this reporter system. Fluorescent reporter systems might be better suited for assays that require monitoring of certain areas of the larvae. However, such assays might suffer from lower sensitivities due to background effects caused by the excitation light, which also poses limits on the detection of fluorescent compounds. Additionally, they might provide less kinetic resolution because of the generally higher stability of fluorescent proteins[23]. Furthermore, they present challenges in terms of imaging equipment, data analysis and data storage that might limit their use for smaller research labs.

The set-up of the GRIZLY assay as a microtiter plate based assay allows for easy integration into typical screening workflows. The assay is easily applicable also in smaller research labs due to its simple handling and data analysis. At the same time, it allows for a substantial degree of automation, *e.g.* automated drug application by pipetting robots or automated distribution of embryos by embryo sorting devices[24,25]. The simple readout does not require automated screening microscopes or sophisticated image analysis software, yet provides a rich set of data on temporal and quantitative aspects of the studied signaling pathway.

In summary, we present a step-by-step protocol for a relatively inexpensive, robust and easy-to-handle chemical screening assay for GC signaling activity *in vivo* and in real time. The assay allows the determination of *in vivo* effects of compounds on GC signaling not detectable in cell culture based assays. Among the many applications for the assay are *e.g.* the determination of genetic effects on glucocorticoid signaling, environmental monitoring of endocrine disruptor effects on glucocorticoid synthesis and signaling activity, and the screening of compounds for unwanted effects on GC signaling or for novel *in vivo* modulators of this important signaling pathway.

Disclosures

We have nothing to disclose.

Acknowledgements

We thank S. Burkhart, C. Hofmann and Simone Gräßle for excellent technical assistance and are grateful to M. Ferg for help with data analysis. We also thank S. Rastegar for critical comments on the manuscript. We acknowledge funding by the Studienstiftung des deutschen Volkes (to MW), the DFG (DI913/4-1) and the Helmholtz Program BioInterfaces at KIT.

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