Functional multiplex reporter assay using tagged Gaussia luciferase

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We have developed a multiplex reporter system to monitor multiple biological variables in real-time. The secreted Gaussia luciferase was fused to ten different epitope tags (Gluctag), each expressed in different tumor cells. By immunobinding of the tags followed by Gluctag detection, this system allowed the independent and real-time monitoring of mixed cell cultures in vitro and of mixed subcutaneous and intracranial tumor subpopulations in vivo.

To study complex processes in systems biology, tools are needed that give insight into multiple molecular and cellular interactions simultaneously and in real-time. Several cellular reporter systems are in use to shed light on biological processes, many based on luciferase enzymes and their photon production upon luciferin substrate addition, including Firefly luciferase (Fluc), Renilla luciferase (Rluc), and Gaussia luciferase (Gluc). These bioluminescent reporters have been used to monitor biological processes such as cellular proliferation and differentiation, transcription factor activity, translational repression by miRNA, and intercellular interactions, reviewed in. Other commonly used reporters are based on the green fluorescent protein (GFP), the red fluorescent protein (RFP) and, more recently, near infra-red fluorescent proteins which can be multiplexed together to monitor several processes simultaneously using spectral unmixing in conjunction with fluorescence molecular tomography. These reporters have several disadvantages including low sensitivity due to autofluorescence, complexity, and the requirement of expensive instrumentation. Further, similar to bioluminescence, limited numbers of variables can be monitored simultaneously, limiting their use in multiplex screening assays and cellular heterogeneity studies. Here, we developed a multiplex bioluminescence assay that allows monitoring of many variables in real-time within the same biological system. This assay is based on Gluc since this reporter is highly sensitive, naturally secreted, and can be detected in the conditioned medium of cells in culture as well as in the blood of animals ex vivo, allowing real-time monitoring of cellular variables.

Results

We constructed a library of lentiviral vectors consisting of the Gluc cDNA fused to ten different epitope tags at its C-terminus, under the constitutively active cytomegalovirus (CMV) promoter resulting in GlucFlag, GlucHis, GlucHA, GlucAcV5, GlucV5, GlucGlu, GlucMyc, GlucKt3, GlucAu1, GlucE2, and the control reporter construct without tag, GlucCtrl. These lentiviral vectors also express cerulean fluorescent protein (CFP) separated from Gluctag by an internal ribosomal entry site (IRES) element as a marker for transduction efficiency (Fig. 1a and Methods section). We employed human U87 glioblastoma cells stably expressing Fluc and the mCherry fluorescent protein (U87-FM) for validation of the Gluctag multiplex system. These cells were transduced with each of the lentiviral vectors to produce ten different cell lines, each stably expressing a different Gluc and CFP (U87-FM-Gluc-GFP). Fluorescent microscopy analysis for mCherry and CFP showed that these cells were nearly 100% transduced with the reporter vectors (Fig. 1b). We first demonstrated that all of the individual U87-FM-Gluc-GFP cells expressed the corresponding tags by immunostaining using specific antibodies, except for GlucAu1, that was not detectable by immunostaining attributed to anti-Au1 antibody properties (Fig. 1c). As a control we stained cells expressing GlucCtrl with the different tag antibodies, which did not show any staining (data not shown). We confirmed Gluc expression by Western blot using anti-tag antibodies (Sup. Fig. 1a) and RT-PCR of the tag region (Sup. Fig. 1b and c). Next, we determined the effect of the tags on Gluc by measuring luciferase activity at different time points in an aliquot of conditioned medium from U87-FM cells expressing...
Figure 1 | Gluc tag multiplex assay development and validation in vitro. (a) Schematic of the lentiviral vector constructs encoding the luciferase reporters. (b) Fluorescence microscopy analysis of a representative U87-FM-Gluc tag-CFP cells (using GlucFlag reporter) showing mCherry levels (in red; marker for tumor cells) and CFP expression (in blue; marker for transduction efficiency). (c) Immunostaining against the various tags in U87-FM cells expressing different Gluc tag. (d) Gluc activity in U87 cells expressing different Gluc tag with respect to cell proliferation over time and cell number. The dashed line represents GlucCtrl activity. (e) Gluc tag immunobinding assay versus total Gluc activity using serial dilutions of the conditioned medium from a 1-day culture of U87-FM cell lines expressing the individual Gluc tag reporters. (f-g) Equal mixture of ten cell lines each expressing a different Gluc tag reporter. (f) Immunobinding assay of Gluc tag from a mixed population. (g) Immunostaining for the ten different tags expression in the mixed population of U87-FM cell culture. (h-i) U87-FM cells expressing all ten different Gluc tag reporters. (h) Immunostaining for the ten different tags in one cell line. The insert panels show staining of control parental cells. Percentages of positively stained cells are indicated. (i) Immunobinding assay of all ten Gluc tag reporters in one cell line after TMZ treatment (black bars) compared to untreated control (white bars). (j) CFP fluorescence microscopy of cells in (h-i) with or without 600 μM TMZ. Size bar (in b, c, g and h) = 200 μm. Size bar (i) = 400 μm. N.d. = not detectable.
each Gluc-tag reporter construct using the Gluc substrate coelenterazine. We demonstrated that fusion of epitope tags to Gluc does not dramatically affect the expression, secretion or activity of Gluc (Fig. 1d and supplemental Fig. 1a–c). We then used the conditioned media containing the different Gluc-tag constructs to optimize antibody-tag immunobinding in a 96-well plate format (Methods). Plates were first coated with monoclonal antibodies against the different tags and incubated with different amounts of conditioned medium allowing Gluc-tag immunobinding. Subsequently, the wells were washed to remove unbound Gluc-tag and subjected to the Gluc assay by direct addition of coelenterazine to the wells and measuring photon counts using a plate luminometer. As a control, an aliquot of

Figure 2 | Gluc-tag multiplex assay in vivo. (a) U87-FM cells expressing different Gluc-tag were implanted subcutaneously in nude mice (n=3) and tumor growth was monitored overtime. Shown are representative Fluc bioluminescence images of the U87-FM-Gluc-tag-CFP tumors expressing the Gluc-tag reporter. (b), Quantitation of the different U87-FM tumors expressing different Gluc-tag reporters; Fluc activity (black), caliper measurement (green), total Gluc activity in the blood (red), and bound Gluc activity after immunobinding (blue). (c–d) U87-FM cells expressing the different Gluc-tag reporters were mixed in equal amounts and injected subcutaneously in the same mouse (n=5). Tumor growth was monitored using Fluc imaging and total Gluc blood assay as in (a–b). Individual cells subpopulation within the same tumor were monitored using the multiplex Gluc-tag assay. (e) Immunostaining for the ten different tags expression in the mixed U87-FM-Gluc-tag-CFP tumor. Black arrows indicate positive staining for cells expressing the corresponding Gluc-tag. Size bar = 200 μm. N.d. = not detectable. (f–g) Mixed population of U87-FM cells expressing all ten different tags were implanted intracranially in the brain of nude mice (n=5). Tumor growth was monitored with Fluc imaging and Gluc total blood assay, and different tumor cell subpopulations were monitored using the multiplex Gluc-tag assay as in (c–d). Fluc bioluminescence imaging of a representative mouse is shown in (f). Data shown in (b,d, and g) as average relative light units (RLU) ± standard deviation. Heat scales in (a, c and f) indicate relative photon levels (photons/sec) as measured by the CCD camera.
conditioned medium was assayed directly for total Gluc activity. We determined that six of the ten individual Gluc tag constructs, i.e. Gluc_{CFP}, Gluc_{HA}, Gluc_{AcV5}, Gluc_{V5}, Gluc_{Au1}, Gluc_{Ctrl}, Gluc_{His}, Gluc_{Myc} and Gluc_{Kt3} reporters showed lower activity upon immunobinding, attributed to suboptimal antibody-tag interactions. In order to demonstrate the use of the Gluctag system for multiplex applications, we mixed equal numbers of the ten different U87-FM-Gluctag-CFP cells and plated them in a single well. One, three and five days later, we measured the total Gluc level in aliquots of the conditioned medium. The levels of the individual Gluc reporters in the mixed culture medium were analyzed by immunobinding with the different tag specific antibodies. We were able to monitor in real-time the growth of the individual U87-FM-Gluctag-CFP cell populations in the mixed culture (Fig. 1f). Immunostaining confirmed the expression of each tag in the corresponding cells in the mixed culture, again except for Gluc_{Au1} (Fig. 1g). In order to determine whether we can measure the activity of ten different Gluctag reporters in the same cell, we transduced U87-FM cells with all ten different lentiviral Gluc reporters. Immunostaining of the U87-FM-Gluc_{OX} tag-CFP glioblastoma cells with tag specific antibodies showed that almost all cells expressed all ten different Gluctag constructs (Fig. 1h). The tag expression as demonstrated by immunostaining is less intense in Fig. 1h as compared to Fig. 1c, which we attribute to the transduction pool of ten different lentivectors used in Fig. 1h. We used these cells to monitor cell viability in the presence and absence of the alkylating agent temozolomide (TMZ), the standard chemotherapy for glioblastoma patients. Gluc reporters immunobinding assays were used to determine the expression of all ten reporters in the presence or absence of TMZ. We treated the U87-FM-Gluc_{OX} tag-CFP cells with TMZ and measured Gluc activity after six days (Fig. 1i). Precipitation of all ten different Gluc reporters showed a ∼75% decrease in Gluc bioluminescence signal after TMZ treatment, similar to the total Gluc signal and confirmed by CFP immunofluorescence analysis (Fig. 1j). Altogether, these results demonstrate that we can measure biologically relevant differences using our multiplex Gluc system.

In order to determine the in vivo applicability of the Gluc reporters, we injected the ten individual U87-FM cell cultures expressing either one of the Gluc reporters subcutaneously in nude mice for singleplex application. In another set, we injected mice with PBS as a negative control (12 groups; n = 3/group). Tumor growth was monitored over time by caliper measurement, and Fluc bioluminescence imaging (Fig. 2a,b). Blood was collected from mice at different time points and 5 μl of whole blood (optimum amount for Gluc blood assay) was assayed for total Gluc activity. At the same time, 5 μl of blood was analyzed for Gluc activity after immunobinding on wells coated with the corresponding tag antibody (Fig. 2b). All of the Gluc reporters, except Gluc_{Au1}, allowed blood monitoring of tumor growth over time in a singleplex assay. We compared the tumor growth as measured by caliper, Fluc imaging and Gluc blood assay to signals obtained from the ten different Gluc reporters after immunobinding (Fig. 2b). The six Gluc reporters Gluc_{Flag}, Gluc_{His}, Gluc_{Kt3}, Gluc_{AcV5}, Gluc_{V5}, Gluc_{Au1} were more sensitive in detecting tumor growth than the others, possibly due to a higher affinity of the antibodies to these tags. To demonstrate the use of the Gluc system for multiplex applications, we mixed equal numbers of U87-FM cells expressing the ten different Gluc and implanted the heterogeneous cell pool subcutaneously in nude mice (n = 5). We monitored tumor growth by Fluc bioluminescence imaging and total Gluc blood assay (Fig. 2c,d). Importantly, we were able to efficiently monitor the growth of the individual subpopulation of the U87-FM cells expressing the different high-efficiency Gluc reporters (Gluc_{Flag}, Gluc_{His}, Gluc_{AcV5}, Gluc_{V5}, Gluc_{Au1}) in the same tumor over time (Fig. 2d). We confirmed the expression of all tags (except for Gluc_{Au1}) in the same tumor by immunostaining (Fig. 2e). To determine the applicability of the Gluc reporters in deep tissues, we implanted orthotopically in the brain of nude mice (n = 5). We monitored tumor growth by Fluc bioluminescence imaging and total Gluc blood assay (Fig. 2f,g). Again, we were able to monitor the intracranial growth of the individual subpopulations of U87-FM cells expressing the high-efficiency Gluc reporters within the same brain tumor in real-time, by blood sampling and the expression of the Gluc reporters in the mixed culture system (Fig. 2g).

**Discussion**

Here we demonstrated the functionality of the Gluc reporters multiplex reporter system by the parallel monitoring of mixed cell cultures in vivo, and of subpopulations in mixed subcutaneous and intracranial tumors ex vivo. The individual epitope tags could also be employed to localize expression by immunostaining. We used ten different Gluc reporters, but this tag library can be readily extended allowing for increased multiplexing. In addition, antibody binding of the low-efficiency tags in the Gluc reporters might be further optimized to achieve higher sensitivity since these tags, except for Gluc_{Au1}, were efficiently detected by immunostaining. Real-time monitoring of individual cells in a heterogeneous mixture may allow, for instance, multiplexed RNAi screening or measurement of drug responses of multiple cell populations in parallel. The Gluc reporters multiplex system might be extended to monitor different variables in a single cell type. For instance, engineering each individual Gluc reporter under the control of different transcription response elements for multiplex transcription factor activity measurements, constructing microRNA (miRNA)-binding sequences in the 3′-UTR of the Gluc reporters for multiplexing monitoring of miRNA activity or by inserting different protease cleavage sites into the Gluc reporters gene for multiplex protease activity measurement. This reporter system offers a versatile tool to study complex processes with different variables in systems biology.

**Methods**

**Cells.** For in vitro and in vivo validation, we used the glioblastoma cell line U87, stably co-expressing Firefly luciferase and mCherry fluorescent protein. Cell lines were maintained in DMEM high glucose complemented with sodium pyruvate, stable glutamine, 10% FBS and pen/strep (all PAA), incubated under standard cell culture conditions of 37°C and 5% CO₂.

**Lentivirus vector construction and transduction.** First, we amplified Gluc by PCR using Accuprime Pfx DNA polymerase (Life Technologies) from Cscw-Gluc-IRES-CFP with specific primers that incorporate an XbaI site downstream of the Gluc cDNA (Forward primer: 3′-GCGGATCCCTCGAGTGACCTGAAAT3′; reverse primer: 3′-CTTCGAGTAGAAGATGTGGTTCACCACTGGCCACCCT-3′). The reverse primer also covers the original stop-codon that is thereby removed. The Gluc cDNA, now containing a unique XbaI site, was ligated back into the Cscw-Gluc-IRES-CFP vector with T4 DNA ligase (Life Technologies), after removal of the wild-type Gluc using Nhel and Xhol (Life Technologies). Plasmid DNA was transformed in XL-10 Gold ultracompetent cells (Aglent Technologies), cultured overnight in LB agar containing 50 μg/ml Ampicillin. We isolated DNA using a DNA plasmid mini kit (Qiagen) and verified successful transformation by XbaI restriction analysis. The Gluc construct was then digested with Xbal and Xhol to insert the different epitope tags. The epitope tags were designed with an Xbal site upstream, a stop codon and Xhol site downstream. A total of 20 μM of both single strand oligonucleotides of the acquired tag DNA (Life Technologies, see Table S1 for sequences) was annealed in annealing buffer (100 mM Tris-HCl pH 7.5, 1.5 mM NaCl and 10 mM EDTA) by heating to 65°C for 10 minutes and slow cooling to room temperature. The epitope tag was then inserted into the vector using T4 DNA ligase and transferred in XL-10 Gold ultracompetent cells. Bacteria were cultured and DNA isolated. We verified the Gluc constructs by sequencing using BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, primer: 5′-GCCAAGTGGAGTGGTGCCT-3′). The Gluc reporters construct was co-transfected with a third generation lentiviral packaging mix (pMDLg/pRRE, pRSV-Rev and pMD2.G, Addgene) in HEK293T cells using Lipofectamine 2000 (Life Technologies). Virus was harvested two and three days after transfection and cell debris was spun down for five minutes at 1,000 × g. U87 cells were transduced overnight with lentivirus using a multiplicity of infection of 100 transducing units per cell in the presence of 8 μg/ml polybrene in standard culture conditions.
Fluorescence microscopy. Successful transduction was verified by visualizing CFP (co-expressed with the Glucmuc construct) and mCherry fluorescent protein (co-expressed with Fluc) using fluorescent microscopy (Leica).

Immunostaining and antibodies. For in vitro immunostaining, cells were fixed in 3.7% formaldehyde for 20 minutes, washed with PBS and permeabilized with PBS containing 0.1% Triton X-100. Cells were then washed three times in PBS containing 5% FBS and blocked with PBS containing 5% FBS. Primary mouse-anti-tag monoclonal antibody (10 μg/ml in 100 μl of PBS, Table S1) was added and incubated for one hour at room temperature. Cells were washed again and incubated with 1 : 100 goat-anti-mouse horseradish peroxidase (HRP) conjugate (Dako) for one hour. After washing, cells were stained with DAB+ (Life Technologies). For tissue section staining, tumors were removed and embedded in paraffin. Microtome sections of 5 μm were deparaffinized in xylene and rehydrated in ethanol series of 100%, 96%, and 70% ethanol. Endogenous peroxide was blocked with 0.3% H2O2 in methanol for 30 minutes. After rinsing with water, antigens were retrieved with citrate buffer (pH 6) with 0.05% Tween 20 using a microwave (Bosch, five minutes 900 watt, 2 mm from the bregma). A total of 2 × 105 cells in 5 ml DMEM was injected intraperitoneally. Imaging was performed with an IVIS CCD camera and analyzed with Living Image software (Caliper Life Sciences). We collected 300 μl mouse blood aliquots from the tail vein in Microvette CB300 EDTA capillary tubes (Sarstedt) for ex vivo whole Gluc measurements and Gluc immunobinding assays.

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Author contributions
JN and TW conceived this study and SVR, BAT, JN and TW designed the experimental setup, with help of DN and WPV. SVR performed most of the experiments. SVR, JN and TW wrote the manuscript, which was edited and reviewed by all the authors.

Additional information
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