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# CHAPTER IV

## Multiplex blood reporters for simultaneous monitoring of cellular processes

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## INTRODUCTION

Secreted blood reporters are valuable tools for sensitive and fast detection, quantification and non-invasive *ex vivo* monitoring of *in vivo* biological processes.[1] Currently, the three most commonly used secreted blood reporters are the secreted alkanine phosphatase (SEAP)[2-5], soluble peptides derived from human carcioembryonic antigen (hCEA) and human chorionic gonadotropin (βHCG)[6-9] and *Gaussia* luciferase (Gluc)[10-14]. The level of these secreted reporters can be measured over time in blood, serum and/or urine to generate multiple data sets without the need to sacrifice the animal, since only a small amount of fluid is required. In contrast to other tools for monitoring of cellular processes, secreted blood reporters are suitable to follow biological parameters along the way, providing new insights in the factors contributing to disease development and progression[1]. During the last 20 years, secreted blood reporters have proven their value in a wide variety of medical fields, including embryo development, viral dissemination, fate of (stem) cells, gene transfer and tumorigenesis and response to therapy.[14-16] The contribution of secreted blood reporters to understanding of these complicated processes would increase even further, if instead of one, multiple parameters could be measured simultaneously and over time.

The discovery of new secreted reporters with different substrate specificities, emission spectra and/or detection assays will allow the development of multiplex assays that are capable in monitoring several processes, given that their separate reactions remain distinguishable. Here, we characterized the naturally secreted luciferase from the marine ostracod *Vargula (Cypridina) hilgendorfi* (Vluc)[17,18] as a blood reporter and multiplexed it with Gluc and SEAP to develop a triple blood reporter system to monitor three distinct biological processes. As a proof of concept, we successfully monitored the response of three different subsets of glioma cells to the chemotherapeutic agent temozolomide[19] in the same animal. This multiplex system can be extended and applied to many different fields for simultaneous monitoring of multiple biological parameters in the same biological system.

## RESULTS

We first characterized a codon-optimized Vluc variant for mammalian gene expression as a blood reporter. Different amounts of U87 human glioma cells stably expressing Vluc and the mcherry red fluorescent protein (RFP) were implanted subcutaneously in nude mice. Three days post-implantation, 5 µl blood samples (in triplicates) were withdrawn, mixed with EDTA (as an anti-coagulant), and assayed for Vluc activity using a luminometer after addition of the vargulin substrate. Several optimization steps for the detection of Vluc in the blood were first performed using different concentrations of the vargulin substrate in blood and serum. We found that 100 µl of 0.25 µg/ml vargulin (diluted in PBS) gave the best signal-to-background (S/B) ratio (supplementary Fig. 1a). Since hemoglobin in whole blood is known to interfere with bioluminescence, we compared the activity of Vluc in blood versus serum and observed that serum gave higher S/B ratio at a concentration of 0.2 µg/ml (supplementary Fig. 1a). EDTA did not have any effect on the Vluc activity (Supplementary Fig. 1b). Using these optimized conditions, we observed that Vluc activity in blood is linear with respect to cell number in a range covering at least 3 orders of magnitudes (Fig. 1a). We then checked the possibility of detecting Vluc in urine, similar to Gluc[14,13]. We found that Vluc activity in urine was also linear with respect to cell number, albeit less sensitive, showing that Vluc is cleared by the kidneys (Fig. 1a). We next evaluated the half-life of Vluc in circulation by intravenously injecting Vluc-containing conditioned medium of cells in nude mice and assaying blood for Vluc activity at different time points. We found that the Vluc half-life in circulation to be around 3 hours (Fig. 1b).

Since it is of uttermost importance that the chemical reaction of the individual reporters used for multiplex application can be distinguished, we determined the specificity of each reporter to its substrate in the blood. U87 glioma cells stably expressing Gluc, Vluc or SEAP (all under the control of CMV promoter) were implanted subcutaneously in different nude mice. Three days later, 5 µl blood was assayed for each reporter activity using coelenterazine, vargulin or SEAP substrate. Significant signal from blood was obtained only when the proper reporter/substrate combination was used, showing no substrate overlap or cross reaction among the

different reporters, and indicating that Gluc, Vluc and SEAP can be used together for multiplex applications (Fig. 1c).

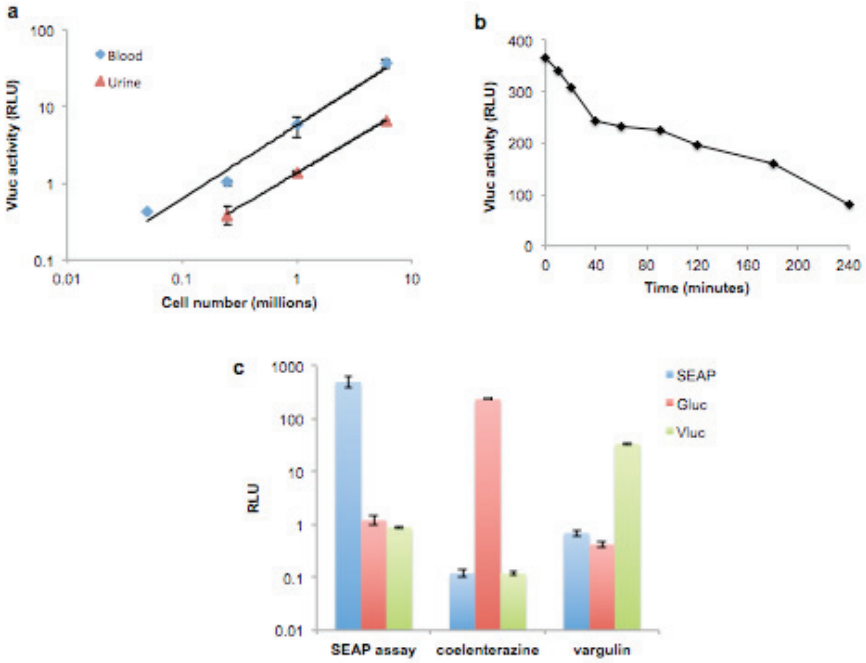



**Figure 1.** Triple secreted reporter system. (a) Linearity of Vluc blood reporter with respect to cell number. Different U87 glioma cells expressing Vluc were implanted subcutaneously in nude mice (n=4/group). One week later, 5 µl blood or urine (in triplicates) was assayed for Vluc activity using 100 µl of the vargulin susbtrate. (b) half-life of Vluc in circulation. Conditioned medium from U87 cells expressing Vluc were filtered, and 150 µl was i.v. injected in nude mice (n=4). At different time points, 5 µl blood (in triplicates) was assayed for Vluc activity. Specificity of each reporter for its substrate. Mice were injected subcutaneously with U87 cells expressing either Gluc, Vluc or SEAP (n=4/group). One week later, 5 µl blood or serum (in triplicates) were assayed for Gluc, Vluc, and SEAP activity. A significant signal is obtained in blood/serum only with the proper reporter/substrate combination.

Finally, we applied the triple reporter system for non-invasive monitoring of three different subsets of U87 glioma cells in response to temozolomide (TMZ), the chemotherapeutic agent of choice for the treatment of grade IV glioma[19]. We used U87 parent cells as well as two TMZ-resistant subclones of these cells, U87R1 and U87R2. U87 parent cells (sensitive to TMZ) were engineered by a lentivirus vector to

express Gluc (U87-Gluc), while U87R1 were engineered to express Vluc (U87R1-Vluc) and U87R2 to express both SEAP and Fluc (U87R2-SEAP/Fluc). Fluc here is used for in vivo localization of U87R2 cells with bioluminescence imaging (since SEAP cannot be imaged in vivo) while Gluc and Vluc are used to localize U87 and U87R1 cells respectively. First, the triple secreted reporter system was confirmed in culture by plating 5,000 of either U87-Gluc, U87R1-Vluc, U87-R2-SEAP/Fluc (singleplex) or combination of all three cells (2,000 cells of each line; multiplex) in a 96-well plate. Aliquots of the conditioned medium were then assayed for each luciferase activity every day over 4 days. Using the singleplex assay, we observed that U87-Gluc cells responded very well to TMZ (>50% cell death) while this drug had no significant effect on U87R1-Vluc and U87-R2-SEAP/Fluc as expected (Fig. 2).

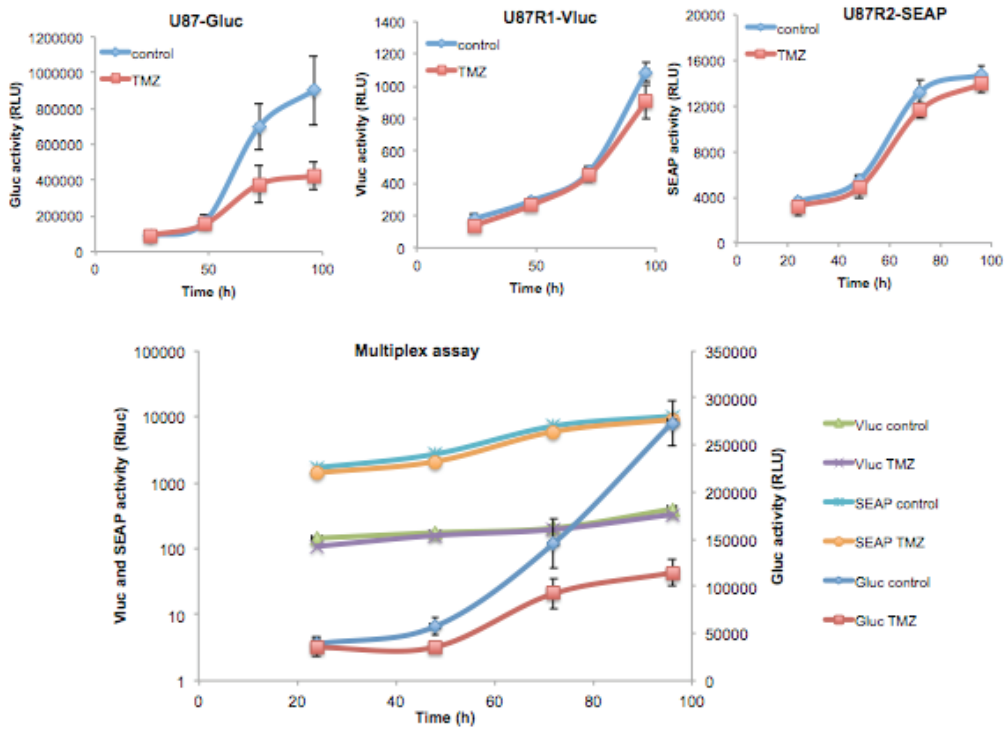



**Figure 2**. Triple secreted reporter system in vitro. U87-Gluc, U87R1-Vluc, U87R2-SEAP/Fluc cells or a combination of all three cell lines (multiplex) were plated in a 96-well

plate and treated with either TMZ (100 nM) or DMSO control. At different time points, aliquots of conditioned medium were assayed for Gluc, Vluc or SEAP reporters. Only U87 parent cells responded to TMZ (as observed by Gluc assay) but not U87R1 or U87R2 cells with both singleplex and multiplex assay.

The multiplex assay showed the exact same phenomena proving that these three reporters can be used together for simultaneous monitoring of three distinct biological processes over time. We then mixed these three cell lines equally and intracranially implanted 75,000 cells (25,000 of each line) in the brain of nude mice. One week later (time zero), a sample of blood was withdrawn and a group of mice (n=6) was injected with 10 mg/kg TMZ, while the other group was injected with DMSO (control). Blood was collected at different time points and 5 µl of blood (for Gluc) or serum (for Vluc and SEAP) was assayed for each luciferase activity using coelenterazine, vargulin or SEAP assay respectively. As expected, a continuous decrease in Gluc level in the blood in response of parent U87 cells to TMZ was observed over time in the treated group, while an increase in Gluc signal was observed in the control group (Fig. 3). On the other hand, both Vluc and SEAP levels increased in serum over time in both treated and control groups showing that U87R1 and U87R2 cells are resistant to TMZ (Fig. 3). Before and 10 days post-treatment, mice were imaged for Gluc, Vluc and Fluc after injection of coelenterazine, vargulin and $_{D}$-luciferin confirming the blood assays (Fig. 3). All together, these results show that Gluc, Vluc and SEAP can be multiplexed together as blood reporters for non-invasive monitoring of biological processes, in real time.

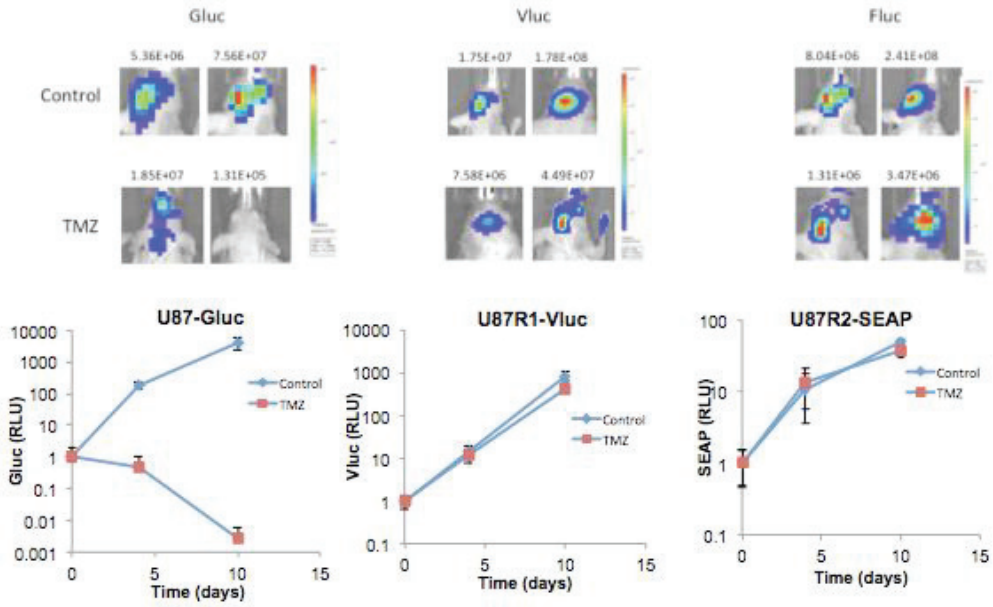



**Figure 3.** Multiplex blood reporter systems. A mixture of U87-Gluc, U87-Vluc and U87-SEAP/Fluc cells (25,000 of each cell line) were intracranially injected in the brain of nude mice. One week later, mice were randomized in 2 groups, which received either 10mg/kg TMZ or DMSO vehicle (n=6/group). Before and at different time points post-treatment, 5 µl blood was assayed for Gluc activity. Likewise, 5 µl of serum was assayed for Vluc and SEAP activity. Before and at day 10 post-TMZ treatment, signal was localized to tumors by in vivo bioluminescence imaging of Gluc, Vluc and Fluc after injection of coelenterazine, vargulin and $_{D}$-luciferin substrates respectively.

In summary, we have developed a multiplexed blood reporter system for simultaneous monitoring of multiple biological parameters in the same experimental animal. This system could be applied in many different fields facilitating the understanding of disease development and expedites findings of novel therapeutics and translation into the clinic.

## EXPERIMENTAL SECTION

*Lentivirus vectors. Lentivirus vectors expressing* CMV-SEAP, CMV-Gluc and CMV-Fluc were previously described[14]. Codon-optimized Vluc cDNA for mammalian gene expression was amplified by polymerase chain reaction (PCR) from pCMV-Vluc

(Targeting Systems) and cloned in a similar vector backbone as CMV-SEAP creating CMV-Vluc. Lentivirus vectors were packaged as previously described.[14]

*Cell culture and reagents.* U87 human glioma cells were obtained from ATCC. U87R1 and U87R2 cells were generated by repeated exposure of U87 cells to low doses of TMZ (a kind gift from Dr. Peter Wesseling, VU University Medical Center, Cancer Center, Amsterdam, the Netherlands). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 100 U penicillin, and 0.1 mg streptomycin (Sigma) per milliliter, at 37 °C and 5% $CO_2$ in a humidified atmosphere. Temozolomide was obtained from Sigma.

*Reporter substrates*. Coelenterazine was obtained from NanoLight™ Technology (Pinetop, AZ) and resuspended at 5 mg/ml in acidified methanol. Vargulin substrate was obtained from NanoLight™ Technology and was resuspended at 5 mg/ml in acidified methanol. $_D$-luciferin was purchased from Gold Biotechnology® (St. Louis, MO) and resuspended at 25 mg/ml in PBS. SEAP was detected using the the Great EscAPe SEAP kit (Clontech) as per manufacturer's instructions.

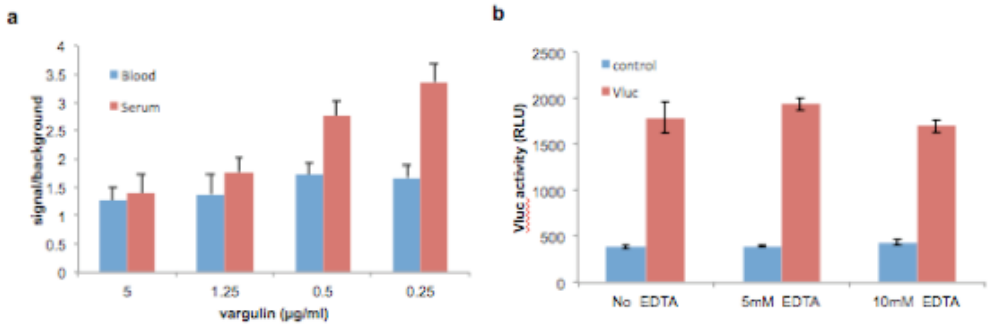
*In vitro experiments.* U87-Gluc, U87R1-Vluc and U87R2-SEAP/Fluc cells were plated in a 96 well plate (5000 cells/well) in a total volume of 50 and 24 hours later treated with either temozolomide (100 nM) or DMSO vehicle. At different time points, 10 µl aliquot (in triplicates) of cell-free conditioned medium was collected and assayed for either Gluc, Vluc or SEAP using either coelenterazine (5 µg/ml in PBS), vargulin (1 µg/ml in PBS) or the Great EscAPe respectively. We refreshed the media of cells 6 hours before measurements to avoid accumulation of the reporter.

*Animal studies and blood collection.* All animal studies were approved by the Massachusetts General Hospital Review Board. U87 human glioma cells, U87R1 and U87R2 were transduced with each lentivirus vector for stable expression by adding the vector directly to the cells using 10 transducing units of each vector per cell. To generate tumors, different amount of these cells (in 50 µl) were mixed with equal volume of Matrigel and injected subcutaneously in the flanks of athymic nude mice. For brain tumor model, a mixture of these cells (25,000 per cell line, in 2 µl

Opti-MEM) were intracranially injected in the left midstriatum of nude mice using the following coordinates from bregma in mm: anterior-posterior +0.5, medio-lateral +2.0, dorso-ventral −2.5. These injections were performed using a Micro 4 Microsyringe Pump Controller (World Precision Instruments, Sarasota, FL) attached to a Hamilton syringe with a 33-gauge needle (Hamilton, Rena, NV) at a rate of 0.2 µL/min. One week later, mice were randomized in 2 groups (n=6/group) and treated with either 10mg/kg temozolomide or DMSO vehicle. Blood samples were collected from these mice as well as from mice with no tumors (negative control) by making a small incision in the tail and directly adding it to an eppendorf tube containing EDTA as an anti-coagulant (10 mM final concentration).

*Ex vivo multiplex blood reporter assays.* For Gluc assay, activity was measured by injecting 100 µl 100 µM coelenterazine (nanolight, pinetop, AZ) to 5 µl of blood and acquiring photon counts over 10 seconds using a luminometer (Dynex). The SEAP chemiluminescence activity was measured in 5 µl serum using the Great EscAPe SEAP kit (Clontech) as per manufacturer's instructions. For Vluc assay, activity was measured in 5 µl blood or serum after injecting 100 µl 0.25 µg/ml of vargulin (diluted in PBS) and acquiring photon counts using a luminometer.

*Triple in vivo imaging.* For Fluc imaging, mice were injected i.p. with 200 mg/kg body weight of $_{D}$-luciferin solution and imaging was performed 10 min later. For Gluc imaging, mice were injected i.v. (through retro-orbital route) with 5 mg/kg body weight of coelenterazine solution diluted in PBS and imaging was performed immediately. For Vluc *in vivo* imaging, mice were injected i.v. with 4 mg/kg body weight (diluted in PBS) and imaging was performed immediately. For sequential imaging of all three reporters, we imaged Gluc on day 1, Vluc 4 hrs later and Fluc on Day 2 to allow enough time for the signal to reach background levels between different imaging sessions. Imaging was performed using an IVIS® Spectrum optical imaging system fitted with an XGI-8 Gas Anesthesia System (Caliper Life Sciences, Hopkinton, MA). Bioluminescent images were acquired using the auto-exposure function. Data analysis for signal intensities and image comparisons were performed using Living Image® software (Caliper Life Sciences).




**Supplementary Figure 1: optimization of Vluc assay.** (a) Blood was withdrawn from mive bearing subcutaneous U87-Vluc tumors and 5µl blood or serum (in triplicates) was assayed for Vluc activity using different concentrations of vargulin (in 100 µl). (b) conditioned medium of U87 cells expressing Vluc or Fluc (control) were assayed for Vluc activity in the presence and absence of EDTA.

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