Multiplexed Profiling of the Cellular Stress Response at Multiple Pathway Points

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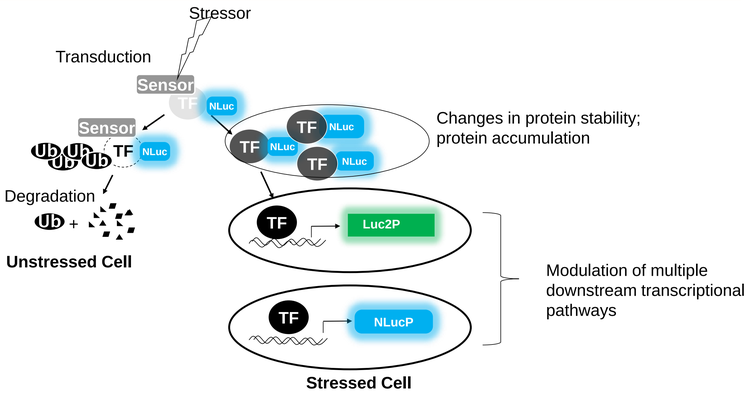
1. Introduction

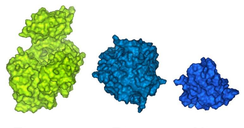
Many cellular responses to changing environmental conditions occur at multiple points in the signaling pathway in order to allow for cellular adaptation and survival. The cellular stress response often involves changes in the stability of a critical regulatory protein followed by downstream modulation of specific transcriptional targets. These changes can occur at sub-cytotoxic exposure levels providing predictive measures of toxicity. Irregular modulation of these pathways can also occur in tumor environments or disease states resulting in undesirable outcomes, presenting potential drug targets.

Dual-reporter assays provide a way to monitor multiple pathway points in the same population of cells. The Nano-Glo Dual-luciferase (NanoDLR) assay allows for sequential monitoring of firefly luciferase (Fluc) and Nanoluc luciferase (Nluc) from the same sample using a simple add-read-add-read protocol. Efficient quenching of the initial Fluc read coupled with the bright Nluc signal give high sensitivity to both reporters. In addition, the NanoDLR assay can be coupled with a fluorescent cell viability assay allowing for a more complete profile of the overall cellular response to be assayed from a single sample.

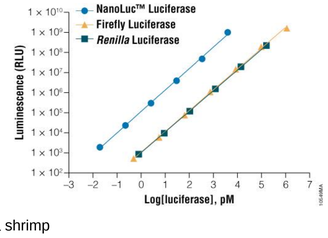
2. Compound Exposure Can Result in a Complex Response at Different Cellular Levels



3. Partnering a Brighter, Smaller Luciferase with Firefly Luciferase for Multiplexed Analysis

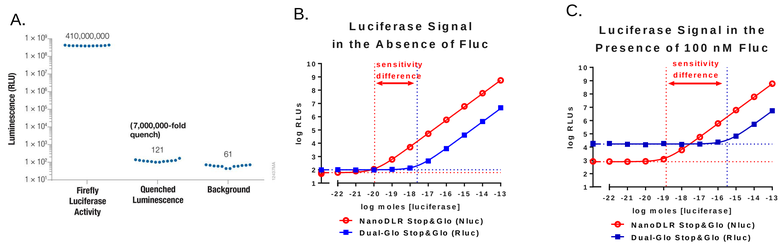


Fluc 61kDa **Rluc 36kDa** **Nluc 19kDa**



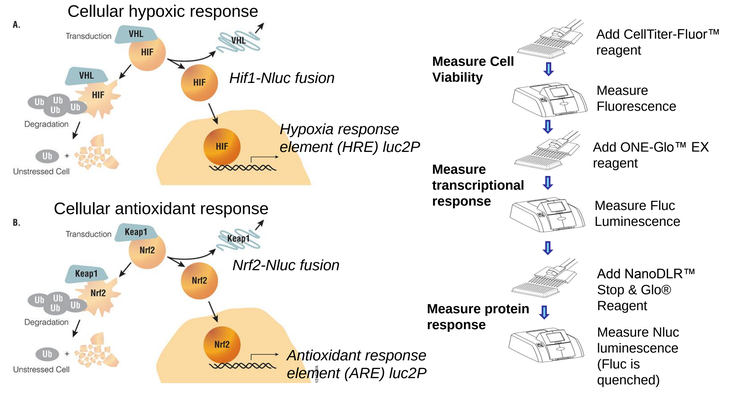
- NanoLuc is 19kDa engineered enzyme from a deep sea shrimp
- Substrates are furimazine and molecular oxygen (ATP-independent)
- About 100-fold brighter than firefly (Fluc) and *Renilla* (Rluc) in comparable assays
- Glow-type signal kinetics only (>2 hour signal half-life)

4. Improved Fluc Quench and Nluc Brightness Give Greater Sensitivity to Nluc in NanoDLR Assay

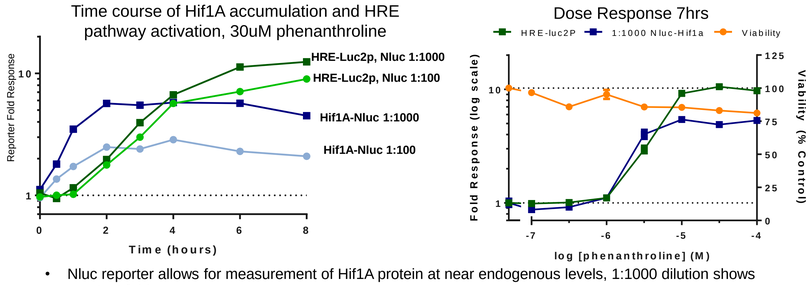


- NanoDLR Stop & Glo quenches Fluc signal to near background levels (Panel A).
- The inherent brightness of Nluc and improved Fluc quenching make the Nluc signal in NanoDLR more than **100-fold** more sensitive than Rluc in Dual-Glo in the absence of Fluc (Panel B). and **over 1000-fold** more sensitive than Rluc in Dual-Glo under high Fluc conditions (Panel C).

5. Workflow for Single-Well Multiplexing, Hypoxic and Antioxidant Responses



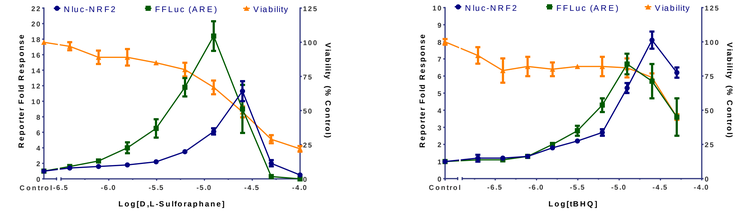
6. Response Profile to Hypoxia Mimetic



- Nluc reporter allows for measurement of Hif1A protein at near endogenous levels, 1:1000 dilution shows greatest fold response.
- Hif1A protein accumulates quickly followed by transcriptional response.
- Both protein accumulation and transcriptional activity show similar dose response, cell viability decreases slightly at highest concentrations. Experiment performed in HEK293 cells transfected with Hif1A-Nluc and pGL4.42[luc2p/HRE] constructs and treated as indicated.

7. Response Profiles to Antioxidant Compounds

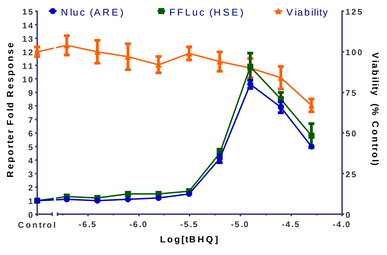
Dose response of NRF2 accumulation, ARE transcriptional response, and cell viability after overnight treatments with D,L-Sulforaphane or tBHQ



- HCT116 cells transfected with pNLF1-NRF2, pKEAP1 and pGL4.37[luc2P/ARE] were treated with D,L-Sulforaphane or tBHQ at indicated concentrations overnight.
- Similar patterns of induction observed with two compounds: D,L-Sulforaphane treatment results in larger fold induction of both NRF2 protein and ARE response.
- Cell viability decreases at highest concentrations of both compounds, D,L-Sulforaphane caused greater impact on cell viability.

8. Profiling Response of Two Cytoprotective Signaling Pathways to Antioxidant Exposure

Dose response of antioxidant (ARE) transcriptional response, heat shock (HSE) transcriptional response, and cell viability after overnight treatment with tBHQ



- HepG2 cells were transfected with pNL[NlucP/ARE] and pGL4.41[luc2P/HSE] and treated with tBHQ overnight at indicated concentrations.
- Both ARE and HSE pathways show similar pattern of induction to tBHQ exposure, both may regulated by NRF2.
- Induction of both signaling pathways occurs before an effect on cell viability is observed providing predictive outcome of cytotoxicity.
- Cell viability decreases at highest concentrations of both compounds, a corresponding decline in both reporters is observed

9. Conclusions

Multiplexing multiple reporters with a cell viability assay allows a more complete profile of cellular response to be assayed from a single cell population.

- Efficient quenching of Fluc signal and brightness of Nluc reporter provide two highly sensitive reporters that can be measured sequentially.
- Multiplexing with a fluorescent reporter assay enables overall health of cells to assessed and aids in the interpretation of the reporter response.
- Assaying multiple points in regulatory pathway can provide greater insight into compound mechanism of toxicity or potential drug mode of action by enabling greater insight into the cellular signaling mechanisms being modulated.
- Assaying multiple signaling pathways provides greater understanding of overall transcriptional responses being modulated by compound treatment.
- Multiplexed analysis can save time and maximizes data output when cells are limiting.

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