

Fluorescent protein biosensors applied to microphysiological systems

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Abstract

This mini-review discusses the evolution of fluorescence as a tool to study living cells and tissues *in vitro* and the present role of fluorescent protein biosensors (FPBs) in microphysiological systems (MPSs). FPBs allow the measurement of temporal and spatial dynamics of targeted cellular events involved in normal and perturbed cellular assay systems and MPSs in real time. FPBs evolved from fluorescent analog cytochemistry (FAC) that permitted the measurement of the dynamics of purified proteins covalently labeled with environmentally insensitive fluorescent dyes and then incorporated into living cells, as well as a large list of diffusible fluorescent probes engineered to measure environmental changes in living cells. In parallel, a wide range of fluorescence microscopy methods were developed to measure the chemical and molecular activities of the labeled cells, including ratio imaging, fluorescence lifetime, total internal reflection, 3D imaging, including super-resolution, as well as high-content screening. FPBs evolved from FAC by combining environmentally sensitive fluorescent dyes with proteins in order to monitor specific physiological events such as post-translational modifications, production of metabolites, changes in various ion concentrations, and the dynamic interaction of proteins with defined macromolecules in time and space within cells. Original FPBs involved the engineering of fluorescent dyes to sense specific activities when covalently attached to particular domains of the targeted protein. The subsequent development of fluorescent proteins (FPs), such as the green fluorescent protein, dramatically accelerated the adoption of studying living cells, since the genetic “labeling” of proteins became a relatively simple method that permitted the analysis of temporal–spatial dynamics of a wide range of proteins. Investigators subsequently engineered the fluorescence properties of the FPs for environmental sensitivity that, when combined with targeted proteins/peptides, created a new generation of FPBs. Examples of FPBs that are useful in MPS are presented, including the design, testing, and application in a liver MPS.

Keywords: Microphysiology systems, fluorescent protein biosensors, high-content screening, fluorescent probes, fluorescent proteins, fluorescence microscopy

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Introduction

This mini-review describes the development and use of fluorescent protein biosensors (FPBs) from a historical perspective and then discusses the use of these tools in microphysiology systems (MPS). MPS aim to reproduce significant human and/or animal organ physiology on a small scale, typically from the milli-human to the micro-human scale, usually based on mass or volume of the organs.^{1–3} MPS integrates multiple cell types representing key organ functions engineered to reflect maximal, native, 3D structure and function in microfluidic devices.^{1,2,4,5} This review will discuss FPBs in the context of MPS, an area of active investigation by the authors. A summary of

FPBs is presented in the context of demonstrations of the design, testing, and use of these tools. It is expected that FPBs will have a major impact on the use of MPS to study normal organ physiology, drug toxicity, and disease models in single organ MPS, as well as in multiple, integrated organ MPS to investigate more complex physiological processes involved in organ interactions.

Fluorescence-based reagents have been used extensively to elucidate and quantify fundamental biological processes within and between cells both *in vitro*^{6–9} and *in vivo*.^{10,11} Our focus in this mini-review is on *in vitro* applications. The use of one category of fluorescence-based reagents, FPBs, to define and quantify the temporal–spatial dynamics of protein functions has been

well established in the literature.⁷ FPBs can be defined as sensors containing two component systems: a sensing domain that recognizes a specific molecular modification or binding partner that is linked to a reporter module that generates the fluorescence signal. Sensing domains can detect specific ligand(s), post-translational modifications, protein-protein interactions, conformational changes, reflect the cellular microenvironment (e.g. pH), and other relevant molecular/cellular processes. The detection of events occurs via altered fluorescence spectroscopic property(s). FPBs can exhibit a change in fluorescence excitation or emission wavelengths, fluorescence intensity, fluorescence lifetime of the excited state, or a change from a non-fluorescent to fluorescent state upon activation or vice versa.⁸

Despite major challenges, the relatively new field of MPS is exhibiting rapid progress.¹ An important goal for the MPS field is to refine, reduce, and ultimately replace the current “gold standard” of animal-based toxicity and disease models that are not fully concordant with human toxic liabilities and disease processes.¹² A major goal is to create a “human or partial human on a chip” that links multiple human organ modules to model key functions such as drug absorption, metabolism, and toxicity. The authors are focused on the implementation of a human liver on a chip and, as part of a broad effort with collaborators, the coupling of the liver with gut and kidney organs on chips. Historically, drug-induced liver injury (DILI) was the most common cause for postmarket pharmaceutical drug withdrawal and continues to be a leading cause of drug attrition.¹³ The potential exists to improve the early identification of DILI that arises from the exposure to toxic substances and intermediates, using MPS models and real-time monitoring of multiple mechanisms of toxicity (MOT), such as alterations in intracellular calcium flux, the generation of reactive oxygen species, and apoptosis.¹⁴ We have developed a human, 3D, microfluidic, four-cell, sequentially layered, self-assembly liver model (SQL-SAL) for studying liver toxicology and disease.¹⁵ Fundamental components of the SQL-SAL include the use of FPBs for real-time analyses of mechanisms of toxicity and disease via high-content screening (HCS) and the integration of a microphysiological system database to capture, analyze, and model data generated within the MPS, in the context of reference data available from external databases).^{*16}

FPBs: A historical perspective

FPBs evolved from an early technology called fluorescent analog cytochemistry (FAC), originally named molecular cytochemistry.¹⁷⁻²⁰ This technology involved the purification of a target protein, the covalent labeling with an environmentally insensitive fluorescent dye, the demonstration of native functions *in vitro*, incorporation of the analogs into living cells through microinjection or bulk loading methods, and then microscopic analysis. In addition, a large number of fluorescent probes engineered through organic chemistry have been developed to measure intracellular

physiological parameters including membrane potential, pH, pCa⁺⁺, and a growing list of metabolites.^{6,7,21}

A variety of fluorescence microscopy methods have been developed to quantify the temporal-spatial dynamics of the fluorescent analogs and fluorescent probes.^{18,22-32} Ratio imaging enabled quantitation without 3D reconstruction of the signals³³ and permitted the quantification of cellular pH, pCa⁺⁺ and other environmental factors, as well as the activation of a variety of biosensors.^{6,7,34-36} Recent advances in light microscopy, such as super-resolution microscopy, go beyond the diffraction barrier to image at greater resolution.³⁷ HCS was developed to create an automated platform to acquire image data and then analyze, display, database, and report on the data from a large number of cells/tissues or even small experimental organisms.³⁸⁻⁴² Significant statistical analyses on large datasets from HCS have demonstrated the critical role of heterogeneity in biological processes and the importance of measuring it in experimental studies.^{43,44} Measuring and interpreting the temporal and spatial heterogeneity in the responses of the MPS disease and toxicity models will be a critical component of investigations using MPS.

A natural progression from labeling proteins with environmentally insensitive fluorescent dyes to quantifying protein dynamics in living cells was to label targeted proteins and/or protein fragments with environmentally sensitive fluorescent dyes^{6,7,44} to measure dynamic chemical and molecular changes in living cells.^{6,34,35,45-48} The reagents, originally named “optical biosensors” have been termed FPBs, involved: the purification of the wild type or site-specific modified protein; site-specific covalent labeling with an environmentally sensitive dye,^{6,48} the demonstration of native functions *in vitro*, including sensing the specific event such as binding ions, metabolites, proteins, and other macromolecules; and then incorporation into living cells through microinjection or bulk loading methods. These early reagents, FAC and FPBs, were difficult to design, construct, and to deliver to cells, but played a critical role in defining a variety of mechanisms of cellular functions and paved the way for the development of the next-generation molecular-based reagents.^{49,50}

The advent of using molecular biology to “label” proteins by fusing the DNA sequence of the protein with the DNA sequence of a fluorescent protein such as green fluorescent protein (GFP) accelerated the use of fluorescence-based reagents in living cells and will be the focus of the remainder of the mini-review.

Genetically encoded FPBs

The discovery and implementation of intrinsically fluorescent proteins, termed fluorescent proteins (FPs), from aquatic species has had a broad impact on the use of fluorescence and FPBs in biological experiments.⁵¹ The GFP, originally obtained from the jellyfish (*Aequorea victoria*) and later derivations through mutagenesis, as well as the discovery of novel target proteins, provided a wide range of fluorescence excitation and emission options for analyzing fusion proteins of interest.⁵² The use of FPs made the creation of fluorescent analogs and FPBs simpler and more powerful.

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Table 1 Major modes of genetically encoded fluorescent protein biosensors (FPBs) with specific examples

Fluorescence mode	FPB type	Specific examples	Reference(s)	
Intrinsic fluorescence	<i>Engineered fluorescent proteins</i>			
		Fluorescent proteins	GFP	118
		Spectral variants	Cerulean & mCherry	119
		Ca ⁺⁺	Pericam, Cameleons	53, 73, 120
		pH	pHluorins, EGFP	121, 122
	<i>Fluorescent protein fusions</i>			
		Complementation	BiFC*	123
		Modified fluorescent proteins	cpFP†	123
		FRET photoconvertible	CFP → YFP FRET	52
		Intracellular localization	PAFPs‡, FRAP, P-PIB§	8, 31, 50, 124
		Intracellular chloride	Clomeleon	125
	<i>Optogenetic biosensors</i>			
		Action potential modulation	Halorhodopsins	65
			Channel rhodopsins	63, 126
			Archaerhodopsins	65
	Voltage sensitive	Mermaid	69	
	Calcium indicators	GECI	66, 127	
Extrinsic fluorescence	<i>Antibody-based systems</i>			
			FAP**	128
			Quench bodies	129
	<i>Covalent labels</i>			
			SNAP-tag, HaloTag	130,131
		FIAsh	9	
		Inteins	132	
		Biotinylation	132,133	

*Bimolecular fluorescence complementation.

†Circularly permuted fluorescence protein.

‡Photoactivatable fluorescence proteins.

§Protein-protein interaction biosensor.

**Fluorogen-activating protein.

Subsequent protein engineering resulted in the emergence of genetically encoded FPBs that could be used to transfect or transduce cells and detect protein-protein interactions based on fluorescence resonance energy transfer (FRET).³¹ Protein engineering of GFP and related FPs has produced distinct FPs with a variety of excitation and emission properties.⁵³ Table 1 presents the major modes of genetically encoded FPBs available and lists specific examples and key reviews related to the class of FPBs. Intrinsic fluorescence modes of FPBs are based on the inherent fluorescence of FPs, while extrinsic fluorescence modes of FPBs are based on the genetic incorporation of the sensing portion of the biosensor and the diffusion of the reporter module into the cells after expression of the targeting portion in the cells. Table 2 lists some commercial sources of standard FPB constructs that can be used to build specific FPBs and provides a starting point for researchers to begin evaluation of suitable candidate FPBs. The broad spectrum of FPs available allow researchers to fuse *de novo* specific protein tags or use pre-existing subcellular tagged FPs to monitor subcellular structures (Evrogen, Addgene, Systems BioSciences Inc.). PAmCherry (Clontech) is an example of a photo-inducible sensor whereby activation with ultraviolet light induces an alteration in the conjugation of the chromophore such that 560 nm light is absorbed and fluorescence emitted at

590 nm.⁵⁴ Various FRET-based FPBs are available to detect post-translational modifications of proteins (Addgene, Clontech, Evrogen). Translocation and receptor modulation sensors can monitor cell membrane dynamics, as has been established for GPCR oligomerization on COS7 cells⁵⁵ (Promega). Various cell cycle and protein-protein interaction biosensors are available, largely based on detection by split GFP (BiFC) or monitoring the production of fluorescent fusion proteins attached to cyclins⁵⁶ (Invitrogen, Clontech). The characteristics of optimal protein-protein interaction FPBs have been discussed in detail elsewhere.⁵⁰ It is also possible to use FPs to create biosensors of gene expression.⁵⁷ However, we limit our discussion in this mini-review to the use of functional protein biosensors.

Subsequent derivations of FPs led to novel photo-dynamic properties and biosensors. For example, the covalent linkage of two FPs with distinct excitation and emission properties produced FPBs with FRET capabilities, known as FRET-based sensors.⁵⁸ These constructs contain a donor and acceptor FRET pair, such that the donor FP when in close physical proximity to the acceptor FP allows for energy transfer from donor to acceptor FP.⁹ This configuration has since been altered for biosensors to monitor conformational changes induced by small molecule ligands/ions (e.g. Ca⁺⁺), protein cleavage sites (e.g. caspases), and

Table 2 Some commercial sources of FPBs

Company	Types of FPBs offered
Evrogen	Ion detection, photoinducible, photoactivatable, subcellular localization, FRET, cell death http://www.evrogen.com/
Invitrogen	Subcellular tracking, cell cycle http://www.lifetechnologies.com/us/en/home.html
Systems BioSciences	Subcellular localization http://www.systembio.com/
Promega	Protein dynamics, subcellular tracking, protein-protein interaction, protein translocation assays http://www.promega.com/
Addgene	Subcellular tracking, various ions and small molecules, protein-protein (FRET) pairs, protein modification https://www.addgene.org/
Clontech	Cell cycle, photoactivatable, photoconvertible, subcellular labeling, protein-protein (FRET) pairs, destabilized proteins http://www.clontech.com/

post-translation modifications (e.g. phosphorylation).⁵⁹ Further experimentation produced hybrid FPs consisting of artificially cleaved and ligated protein configurations. For example, the circularly permuted FPs create new amino and carboxyl termini by cleavage which results in altered fluorescence spectra. The circularly permuted fluorescent proteins have been used to create additional biosensors for Ca^{++} .⁶⁰

Specialty FPBs

A number of fast kinetic, genetically encoded light sensitive proteins have been developed that can reversibly activate, silence, or report on molecular processes of the action potential and other cell functions in neuronal cells and circuits. Halorhodopsins (chloride channel), channel rhodopsins (light gated ion channel), and archaerhodopsins (proton pump) are examples of photoactivatable proteins that are inserted into the genome to control neuromodulatory events *in vitro* or *in vivo*.^{61–65} Other FPBs have been engineered to monitor the rapid molecular processes associated with chloride and calcium ion modulation, as well as voltage-sensing membrane proteins.^{65–69} These FPBs allow long-term, non-invasive imaging of neuronal and other cell types by converting physiological signals into measureable changes of intrinsic fluorescence on the order of milliseconds to seconds. Furthermore, the FPBs developed to modulate or monitor neuronal activity can be encoded into the genome by a variety of viral and non-viral methods, and hence, can be successfully used in mammalian and non-mammalian cells for targeting specific cell populations.^{61,66,68}

Intrinsic engineered FPs include spectral variants of GFP, FP fusions used to monitor protein dynamics and post-translational modifications as well as calcium and

pH biosensors. Engineered fluorescent fusion proteins include photoactivatable (PAFPs) or photobleachable Fluorescence recovery after photobleaching (FRAP) protein fusions that undergo spectral changes when exposed to a particular wavelength of light and are used to track protein dynamics.^{70,71} Potentially interacting proteins can be investigated with bimolecular protein complementation (BiFC) linking proteins under investigation to split domains of FPs.⁷² Intracellular calcium quantitation includes the ratio-metric, FRET-based cameleon biosensor utilizing calmodulin and an M13 calcium sensing domain.⁵³ The calcium sensor pericam also provides reversible calcium sensing using a protein scaffold that is based on circularly permuted GFP.⁷³ The EGFP derivative of GFP, and subsequently the pHluorins, allow for pH measurements in the cytosol and other organelles with spectral shifts occurring at different pH ranges.^{74,75}

Extrinsic FPBs are covalent or non-covalent protein labels that are not inherently fluorescent but selectively bind fluorescent molecules. The FAsH and HaloTag, for example, are peptide fusion tags that bind different fluorescent reagents and may be advantageous when larger FP fusions perturb protein function or localization. Fluorogen activating proteins (FAPs) permit protein localization, dynamics, and pH studies through fusions of proteins of interest to single-chain antibodies that bind fluorophores with low background fluorescence such that the fluorophores exhibit a large increase in fluorescence signal only when bound to the FAP.⁷⁶ FAPs also do not require washout of excess reagent prior to imaging due to the low fluorescence background of the fluorophore when not bound to the FAP protein fusion.⁷⁷

FPBs versus the use of diffusible fluorescent probes in MPS

An exciting next frontier for FPBs is their application in MPS. There are several advantages to the use of stable FPBs in MPS compared to widely used diffusible fluorescent probes.²¹ Most fluorescent probes are not targetable to specific cells and therefore cannot be directed to a certain cell type, or specific subcellular compartments in only those cells, within a complex, multicellular system such as that found in an MPS. The lack of cell type targeting may lead to non-specific interference from neighboring cells when performing experiments. A further limitation of diffusible fluorescent probes is the relatively short half-life in cells. The fluorescent probes are pumped out or sequestered and must be re-added to the cells when studying the MPS over extended times. Another consequence of using diffusible fluorescent probes is the relatively high cytotoxicity induced upon excitation. Phototoxicity can occur within cells due to the generation of reactive oxygen radicals.⁷⁸ Most of the fluorescent probes exhibit greater phototoxicity when compared to FPs exposed to the same dose of light (Unpublished observation). Light-induced cell damage is of particular concern in MPSs that aim to function for several weeks in order to monitor chronic toxicities or disease states. The critical role of minimizing the dose of illumination in live cell studies has been discussed elsewhere.⁷⁹

Table 3 Methods for the incorporation of genetically encoded FPBs into cells

Technique	Advantage	Limitation	Review(s)
Transient transfection	Rapid	Temporary expression Low efficiency Cytotoxicity	83
Virally mediated transfection	Primary cell compatible Transient or stable	Random integration Cytotoxicity	85
Electroporation-based methods	High efficiency Primary cell compatible	Cytotoxicity Expensive	84
Knock-in	Targeted and stable gene insertion Replaces native gene	Time consuming Requires significant expertise	89
Gene editing	Targeted and stable gene insertion Replaces native gene	Time consuming Technically challenging Expensive	87, 88, 95

In contrast to diffusing fluorescent probes into cells, FPBs that are genetically encoded can be targeted to specific cells and subcellular compartments before assembling multiple cell types into the MPS. Integration of the FPBs into the cellular genome allows the expression of the biosensors over extended times. Inducible systems or gene-specific promoter elements can also be used to “turn on” the expression of the selected biosensor, increasing the control of the measurements. Some examples of inducible gene expression include the Tet-On system (Clontech) that allows for inducible gene induction or repression in response to doxycycline in the culture media.⁸⁰ This system has been recently expanded upon in an effort to create lentiviral delivery of the reverse tetracycline-controlled transactivator to ease development of the transactivator cell line.⁸¹ Gene-specific promoter elements can be placed upstream of the FPB encoding region for cell-type specific temporal and spatial expression.⁸² Such molecular approaches can then be targeted into a subpopulation of cells within the overall MPS device. The subpopulation of cells in an MPS that have been transduced with FPBs has been termed “sentinel cells.” The sentinel cells allow for real-time monitoring of key physiological events and the development of specific molecular signatures.¹⁶ The variety of different excitation and emission properties of FPB and FP allows a great deal of flexibility in terms of multiplexing different sentinel cells within an MPS.

Methods to deliver FPBs to cells

A key step in biosensor implementation is determining the optimal method of delivery into target cells to maximize efficiency and minimize toxicity or other side effects. Several examples of these methods are summarized in Table 3. For initial studies designed to test the newly created FPBs, easily cultured and transfected cells can be transiently transfected.⁸³ However, not all cell lines are amenable to chemical transfection nor will the delivery gene be integrated into the host cell genome in the absence of some of selection procedures.⁸⁴ Viral delivery methods, such as adenovirus transduction, are often used for primary or otherwise difficult cell lines.⁸⁵ Lentiviral particles can be

produced with the FPB construct of interest whereby the FPB is randomly inserted into a target cell genome. The advantage of using the lentiviral approach is that most cell lines, primary cells, and induced-pluripotent stem (iPS) iPS-derived cells can be transduced for evaluation of new FPBs.⁸⁶ The use of site-specific gene targeting has been a topic of great interest recently and is discussed in detail below in regards to iPSC.^{87,88,89}

Case study: Implementation of FPBs in the liver MPS (SQL-SAL)

Strategy of testing functions of FPBs in cells

The successful application of FPBs involves a phased approach of implementation and validation (Figure 1). This approach must balance practical as well as functional parameters of the chosen FPBs for testing and eventual use in MPS. The phased approach for this purpose begins by testing the function of FPBs in relevant cell lines grown in static cultures in microplates starting with the transfection of the cells with the FPB construct. The best candidate FPBs suited for a particular MPS are then chosen including any further molecular engineering of the constructs. The FPBs are then tested and validated in human primary cells in static culture using the lentiviral delivery system, since this system can transduce many types of cells. As a final step, induced-pluripotent stem cells matured to the selected cell types and transduced with the lentiviral system are tested. The use of iPSC is advantageous for two reasons: (a) provides unlimited source of otherwise expensive, difficult to obtain, and/or variable quality human primary cells; and (b) allows for the use of patient- or disease-specific cells for study. iPSC can also be modified by targeted genetic manipulations.^{90,91}

The development of MPS systems can take advantage of cell lines that are easy to grow and are less expensive to test the engineering, biocompatibility of materials, robustness, and reproducibility in the context of the requirements for high-quality imaging. Cell lines are also useful for testing and characterizing the quantitation of FPB readouts as dictated by the particular application of the MPS. Initial work

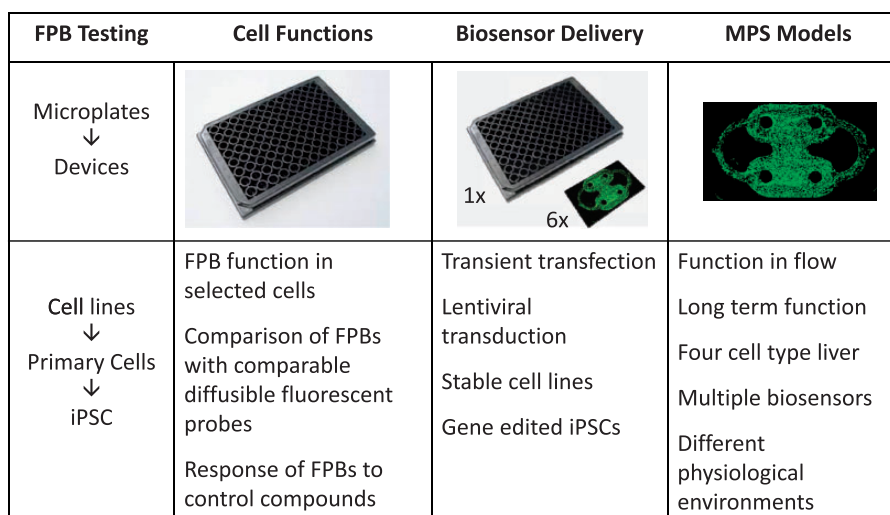


Figure 1 Diagram of the implementation of fluorescent protein biosensors (FPBs) in microphysiological systems (MPS). The first phase involves testing of the function of FPBs in immortalized cell lines, primary cells, and induced pluripotent stem (iPSC) cells in static plate cultures that utilizes transient or stable gene delivery methods. The second phase involves optimal delivery to primary cells and iPSC in both microplates and microfluidic devices (microfluidic device is enlarged 6x relative to the microplate for visualization purposes). The final stage involves the incorporation of the FPBs in MPS models that include multiple cell types, media perfusion, and multiplexing of different FPBs to model distinct physiological environments and disease states for long-term function.

performed in our lab used immortalized cell lines, such as HepG2, starting in static culture to determine the optimal strategy to validate the FPBs for HCS. The rationale for this approach was to test specific FPBs, in a cost-effective and less time-consuming way, for use as live-cell sensors (“sentinel cells”) that can potentially give predictive data of compromised liver health and function. Investigations can then be performed in static plate cultures of primary human cells, in our case primary human hepatocytes, to confirm the organ-specific utility of a particular FPB. Subsequent validation in the relevant primary cells can be performed with drugs and biological modulators with known mechanisms of action.

Finally, the use of FPBs in MPS allows for closed loop testing. Closed loop testing is the process by which the readouts from the system are used to modulate the experimental protocol. For example, the real-time readout of ROS can be used to maintain a low level of ROS by adjusting the test compound concentration, allowing a better determination of the chronic effects of ROS generation. Although a follow-up study can be initiated using a concentration optimized from an earlier study, the advantage of live readout of FPBs allows for real-time dosing adjustment during the ongoing study for a specific measurement such as defining the no observed adverse effect level, the maximum tolerated dose, or the optimal concentration for drug efficacy or disease treatment.

Optimizing delivery of FPBs into cells

The second phase of implementation is optimizing the delivery of FPBs. As a case study for using a stable lentiviral gene delivery system for FPB incorporation into MPS, primary human hepatocytes were investigated. The use of primary human hepatocytes in the SQL-SAL is preferred over cell

lines due to their retention of liver metabolic and clearance functions *in vitro*. Figure 2 shows an example of steps required to incorporate a commercially available FPB construct, in this case the HyPer FPB, into a lentiviral expression vector. After testing and initial studies in HepG2 liver carcinoma cells confirmed the ROS sensitivity, the lentiviral supernatant was incubated with primary human hepatocytes. Transduced hepatocytes were then used for validation studies and subsequently incorporation into MPS models. The efficiency of transduction ranged from ca. 30 to 60% in human primary hepatocytes and was higher in HepG2 cells.

The reproducibility of delivery of FPBs into cells would improve with targeted insertion rather than random FPB integration into the genome. Considering the technical challenges of targeting the insertion site on the host genome in primary cells, it is likely such methods are best deployed with iPSC planned for the final design of an MPS. The latter would provide a renewable resource of cells with an FPB of interest that could be differentiated into the required adult cell type. Knock-in strategies may be used to perform specific gene targeted insertions, using Cre/lox methods for gene targeting.⁸⁹ More recently, gene editing methods of zinc-finger nucleases,⁹² TALENs,⁹³ and CRISPR-based editing⁹⁴ have all been used to modify iPSCs. These methods are evolving rapidly and allow for targeted insertion events that do not leave extensive non-gene-associated flanking DNA sequences, thereby decreasing the size of the insertion and reducing any potential interference from insertion of non-gene DNA.

When comparing CRISPR-Cas9 gene editing to lentiviral gene delivery, lentiviral-based methods are more straightforward but with a higher potential for confounding artifacts due to random insertions. Gene editing techniques, while growing in popularity, require careful controls to be

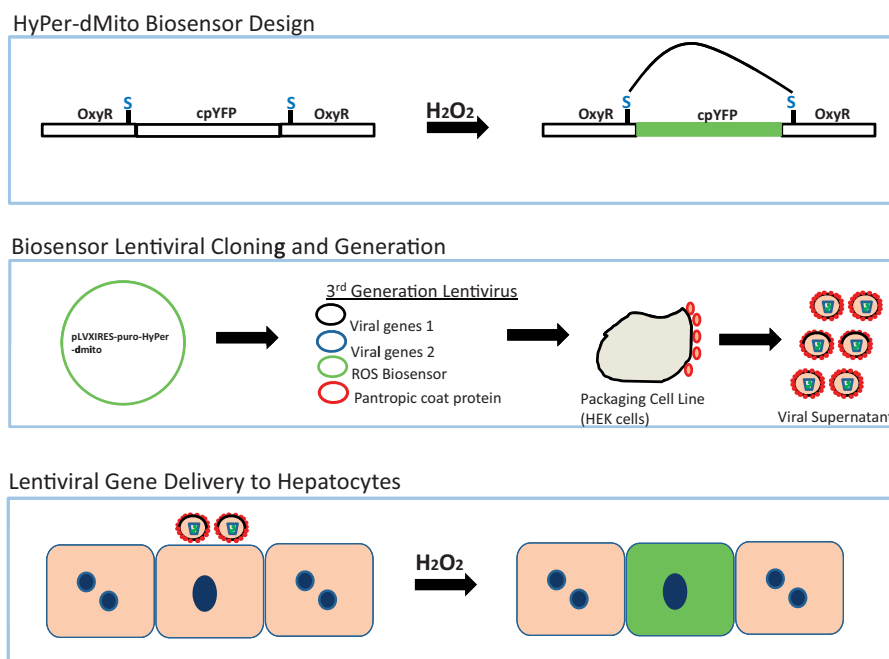


Figure 2 Schematic representing steps required for generation of lentiviral packaged FPBs. The top panel shows the concept of the biosensor design of the hydrogen peroxide sensing FBP (Hyper-dMito, Evrogen) with the cpYFP inserted into the oxidation sensitive bacterial transcription factor, OxyR. In the presence of hydrogen peroxide a disulfide bridge is formed in OxyR, resulting in fluorescence signal from the cpYFP. The middle panel describes the subcloning of the HyPer-dMito into a lentiviral vector and lentiviral packaging. The lower panel indicates lentiviral supernatant incubated with primary human hepatocytes to produce a subpopulation of hepatocytes stably expressing the Hyper-dMito. The subset of cells containing a FBP to provide live cell readouts is referred to as sentinel cells.

performed to screen for off-target effects of FPB insertion.⁹⁵ This has been of particular concern with CRISPR-Cas9, due to the small base pair nuclease recognition site that has the potential for off-target cutting.⁹⁶ Good experimental design can reduce the potential for off-target effects. For example, multiple CRISPR-Cas9 nucleases can be designed for a single target cut site and two or more separate cell lines can be carried forward into development and phenotype screening,⁹⁷ or double-nicking nucleases can be used to increase the cleavage recognition site.⁹⁸ These efforts may vary in their success rate and thus delay progress of FPB incorporation into iPSCs, making these efforts worthwhile only after demonstration and validation of physiological responses. Taken together, and until iPSC knock in lines are more readily available, lentiviral gene delivery is a rapid, stable, and reliable method to deliver FPBs across many cell types. The lentiviral delivery also allows experiments to be performed when time and cost are at a premium.

Validation of FPBs for liver functions

Figure 3 depicts the validation of the HyPer-dMito ROS biosensor (Evrogen) transduction of HepG2 cells. Drugs known to induce ROS production were used to test both a standard diffusible fluorescence probe for ROS, as a positive control, and the FPB. Hepatocellular damage is known to occur in response to the generation of mitochondrial reactive oxygen species.⁹⁹ Mitochondrial ROS detection in both parenchymal and non-parenchymal cells is therefore an important molecular signature for early indication of

liver damage. Dihydroethidium (DHE) reacts with superoxide radicals and the resulting product is a red-fluorescent, DNA binding 2-hydroxyethidium¹⁰⁰ that intercalates into DNA.¹⁰¹ Either DHE was added to the media and diffused into the HepG2 cells, or the cells were transduced with a lentiviral HyPer-dMito FPB, and the cells were treated with either vehicle or a liver toxic drug (nefazodone or troglitazone). HCS was used to quantify the generation of ROS. Troglitazone and nefazodone, but not vehicle, produced ROS species as detected by increased DHE fluorescence (Figure 3(a)). The attenuated nefazodone response, compared to troglitazone, is due to both DHE substrate depletion,¹⁰² as well as a reduction in nefazodone-generated ROS in metabolism-deficient HepG2 cells. The HyPer-dMito FPB exhibited ROS-induced fluorescence when challenged with troglitazone and nefazodone comparable to the response observed with DHE (Figure 3(b)). The HyPer-dMito FPB can be measured over at least 28 days and is reversible with drug washout (data not shown), while DHE diffuses out of cells over time and is not reversible. We found that analysis by ratiometric or single wavelength emission exhibited increased fluorescence signal versus vehicle control (Figure 3(b)). See supplemental materials for details.

The HyPer-dMito sensor can be used in live cell experiments to monitor generation of mitochondrial hydrogen peroxide.³⁶ Importantly, this FPB is sensitive to low concentration ranges of liver toxic drugs, including troglitazone, similar to what has been previously described. Ratiometric imaging can be used to control for heterogeneous biosensor

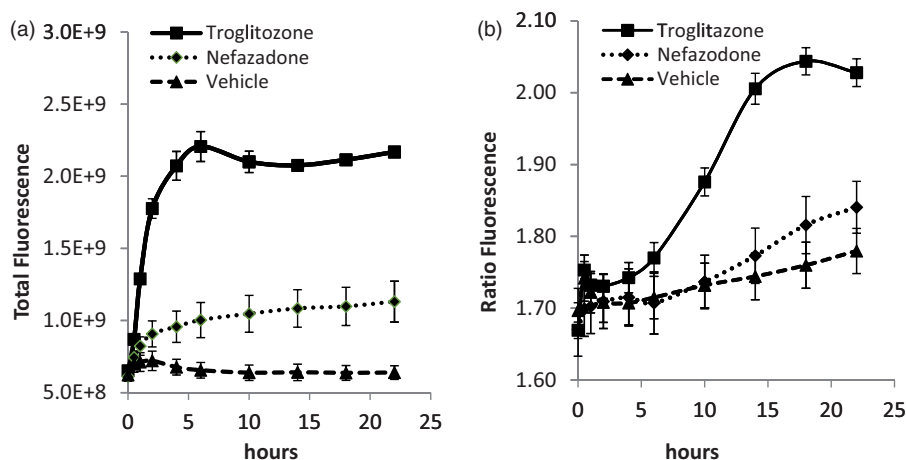


Figure 3 Determination of mitochondrial ROS induction by hepatotoxic drugs. (a) Monitoring ROS production with a standard diffusible probe, dihydroethidium (DHE) fluorescence, in HepG2 cells in static culture treated with 60 μ M troglitazone, a validated drug that induces ROS, 60 μ M nefazodone that also induces ROS production, and 1% DMSO in media (vehicle). Error bars are \pm standard error of the mean on duplicate wells and fields. All curves are normalized to the initial time 0 value. (b) Mitochondrial ROS production (hydrogen peroxide measured with the HyPer-dMito biosensor fluorescence) in lentiviral transduced HepG2 cells treated with 60 μ M nefazodone, 60 μ M troglitazone, and vehicle. Data are presented as total fluorescence or ratiometric values of 488/405 nm. Error bars are \pm standard error of the mean on duplicate wells and fields

expression within a population of cells and the potential for pH effects. Use of only the 488 nm excitation, however, does indicate overall generation of hydrogen peroxide. The use of the single wavelength excitation might be useful to limit phototoxicity within a MPS that must be viable for several weeks and a relative measure of ROS change is sufficient.

Apoptosis is an important MOT that can result in hepatic injury. Loss of hepatocytes, as well as the programmed cell death of liver non-parenchymal cells such as Kupffer cells has been associated with DILI.¹⁰³ Early determination of hepatocellular damage and apoptosis would provide better insight into the relevant MOT of drug treatment under investigation.¹⁰⁴ Mitochondria are a key organelle during the induction of apoptosis, as the loss of the mitochondria membrane potential and integrity can result in the release of pro-apoptotic factors such as cytochrome c.¹⁰⁵ Additionally, mitochondrial calcium fluxes are associated with the onset of apoptosis, as well as additional liver cell pathology.¹⁰⁶ Three FPBs provide three different indications of the induction of apoptosis: mitochondrial calcium uptake, cytochrome c release, and caspase-3 activation. These three FPBs were evaluated in HepG2 cells and compared to the potentiometric mitochondrial probe tetramethylrhodamine ethyl ester (TMRE) or titrations of calcium into calcium free media (Figure 4).

Case12 is a FPB that indicates cytosolic or mitochondrial calcium concentrations depending on the targeting peptide engineered into the FPB and can be applied as an apoptosis FPB.¹⁰⁷ The reversible FP provides sensitivity to nanomolar concentrations of intracellular or mitochondrial calcium. It is based on a circularly permuted yellow fluorescent protein modified with the calcium-binding domain of calmodulin and an M13 peptide. The biosensor is not fluorescent until calcium ions bind and induce a conformational change that restores the fluorescence emission of

the FP chromophore.¹⁰⁸ Case12-transduced HepG2 cells responded to titrations of calcium into media as well as the hepatotoxins troglitazone and nefazodone (Figure 4(a)), compounds known to increase cytosolic calcium.¹⁰⁹

Another apoptosis FPB uses a mitochondrial targeting sequence derived from cytochrome c oxidase VIII linked to copGFP (Systems Biosciences Inc.) that is a surrogate for cytochrome c release from mitochondria. HepG2 cells transduced with the biosensor packaged in a lentiviral delivery system (Figure 2) indicate a decrease in the mitochondrial fluorescence following treatment with nefazodone and troglitazone but not buspirone or trazodone (Figure 4(b)).

Finally, the FRET-based casper-3BG (Evrogen) FPB is specific for caspase-3 cleavage, with improved efficiency.^{110,111} The biosensor construct is comprised of two covalently linked FPs, TagBFP and TagGFP2, containing the caspase-3 amino acid cleavage recognition sequence DEVD in the linker (Evrogen). An increase in the expression and activation of caspase-3 results in a loss of FRET in the casper-3BG biosensor and an increase in 450 nm fluorescence. Nefazodone and troglitazone are compounds reported to activate caspase-3 in human liver cells^{112,113} but not buspirone or trazodone in agreement with our results (Figure 4(c)). The results can be compared to the loss of the mitochondrial potential, as indicated by TMRE fluorescence, with nefazodone and troglitazone, but not buspirone or trazodone (Figure 4(d)).

Table 4 lists some of the commercially available, genetically encoded FPBs constructs tested in our liver MPS program and Table 5 demonstrates the validation of the biosensors, using standard screening statistics. Each of these probes in combination with real-time, live cell imaging provides data for determining MOT for toxicological assessment.^{40,114}

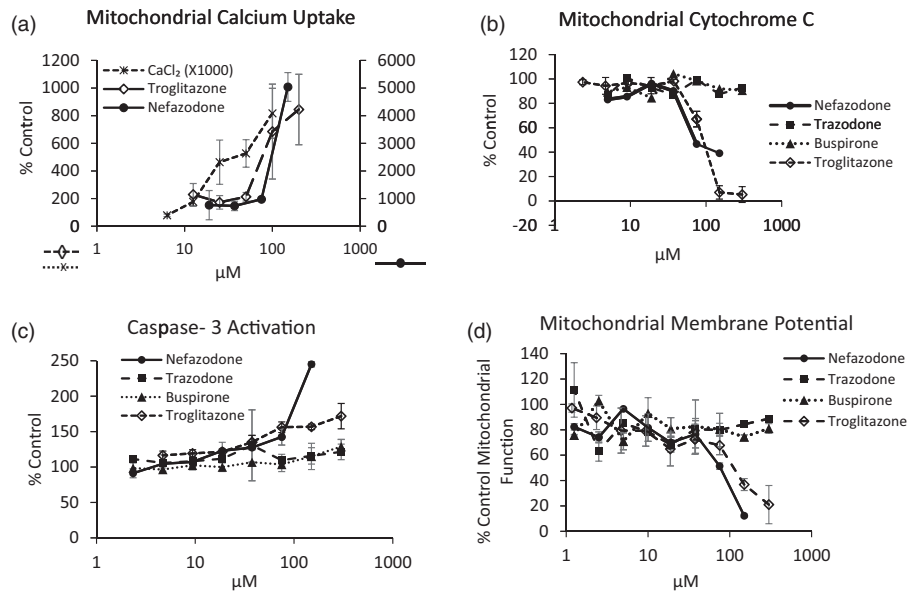


Figure 4 Steps in the induction of intrinsic apoptosis established through the use of three genetically encoded biosensors and the diffusible probe TMRE in HepG2 cells in static culture. (a) Apoptosis induction resulting from mitochondrial calcium uptake measured by the biosensor Case12-mito (Evrogen). Mitochondrial free calcium is increased at 16 h following addition of CaCl₂ in microplates containing Case12-mito transduced HepG2 cells or by addition of the hepatotoxins nefazodone (2nd axis) and troglitazone. (b) Apoptosis arising from mitochondrial damage is monitored at 16 h in HepG2 cell transduced with the cytochrome c-GFP biosensor in response to nefazodone and troglitazone but not the non-hepatotoxins trazadone or buspirone. (c) Apoptosis induction monitored at 16 h by the activation of caspase-3 in HepG2 cells transduced with the Casper-3BG (Evrogen) FPB by nefazodone and troglitazone but not trazadone or buspirone. (d) Mitochondrial membrane potential in HepG2 cells with TMRE (200 nM) for 1 h demonstrates the decrease in mitochondrial membrane potential as a loss of 605 nm fluorescence under increasing nefazodone and troglitazone dosing. Values represent mean and SD of triplicate measurements

Table 4 Selected commercially available, genetically encoded FPBs used in liver MPS

FPB	Source	What it indicates	References
HyPer-dMito	Evrogen	H ₂ O ₂ concentration	36
Case12-Mito	Evrogen	Mitochondrial calcium uptake	107
Casper3-BG	Evrogen	Caspase-3 activity	110
pCT-Mito-GFP	Systems BioSciences	Mitochondrial membrane integrity	134
pCT-H2B-GFP	Systems BioSciences	Cell localization	134

Table 5 Validation of FPBs in primary hepatocytes

FPB	FPB colors	Control compound	Conc (μM)	SSMD
Nuclear/cell position (Histone H2B-FP)	Blue	Menadione	100	>2 (at 5 h)
	Green			
	Red			
Apoptosis (cytochrome C oxidase VIII subunit tagged-FP)	Green	Menadione	100	>2 (at 5 h)
	Red			
Mitochondrial reactive oxygen species (HyPer-dMito)	Green	Menadione	100	>2 (at 2.5 h)
Mitochondrial calcium uptake (Case12-mito)	Green	Menadione	50	>2 (at 4.5 h)

The early success of monitoring liver-specific MOTs has encouraged ongoing efforts in our lab to develop new biosensors to monitor additional crucial liver cell pathologies, such as general oxidative stress fluctuations and non-alcoholic fatty liver disease, to name two.

Example of FPBs used in a liver MPS device

The final phase in the development and application of FPBs is the incorporation of the “sentinel” cells that contain the FPBs into an MPS organ model. Figure 5 demonstrates the functioning of the ROS FPB in a two-cell type (hepatocyte

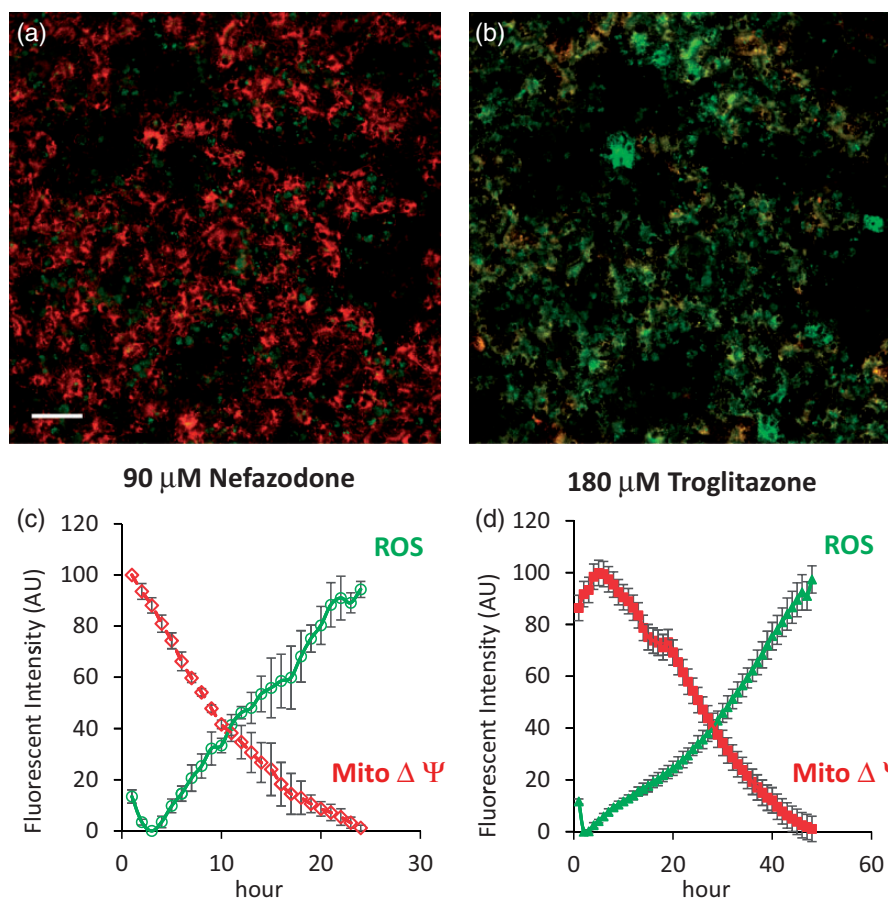


Figure 5 Demonstration of HyPer-dMito FPB activity in a human two cell liver MPS model. (a) Image of TMRE fluorescence and HyPer-dMito fluorescence at time 0 in human primary hepatocytes in a microfluidic device also containing endothelial cells. Image is dominated by TMRE (red) from active mitochondria (white bar = 50 μm). (b) Image of TMRE fluorescence and HyPer-dMito fluorescence in the same field after 20 h exposure to nefazodone (90 μM). Image is dominated by HyPer-dMito (green) following loss of mitochondrial membrane potential and the generation of ROS. (c) Time course of induction of ROS and loss of mitochondrial membrane potential resulting from exposure to nefazodone (90 μM). (d) Time course of induction of ROS and loss of mitochondrial membrane potential resulting from exposure to troglitazone (180 μM). Values are linearly scaled from minimum to maximum and represent the mean \pm SD of nefazodone (n=3) or troglitazone (n=4) images.

and endothelial cell) human liver MPS treated over a 24 h period with an acute dose of nefazodone or troglitazone, showing the loss of the membrane potential followed closely by the increase of the ROS production. The FPBs remained functional over at least 28 days.¹⁵

Pitfalls/precautions in using FPBs

FPBs are powerful tools for real-time monitoring of physiology *in vitro*. However, care must be taken to ensure proper controls are performed such that the interpretation of the data is not influenced by artifacts. Many of the FPBs are well documented to be sensitive to perturbations in pH, thereby producing changes in their fluorescence spectra. The HyPer-dMito (ROS) FPB, for example, has been a subject of debate in the literature with regards to increases in the fluorescence measured at a single excitation wavelength that may be attributable to changes in pH.¹¹⁵ Controls for pH changes can include the use of diffusible probes or genetically encoded pH sensing FPBs with different spectral properties from the functional biosensor of interest.¹¹⁶ For example, we compare performance of FPBs

in static microplate cultures with pH sensitive, diffusible probes (e.g. SNARF-1AM, Molecular Probes, data unpublished). Useful discussion related to proper controls and imaging of diffusible probes has been published.¹¹⁷ Many drugs are fluorescent and the fluorescence level of the drug in the cells without a biosensor can be used to correct for the background, provided it is identified before the experiment. It is advisable to perform compound or drug dosing studies with the cells of interest in microplates first and to optimize imaging conditions to identify drug fluorescence before investing in the use of MPS organ models.

Future of FPBs in MPS

It is clear that real-time readouts of key cell/tissue/organ functions will be an important component of the MPS models. There are a number of valuable FPBs that are already available from commercial suppliers (Table 2) and this is an important starting place for investigators. New FPBs developed for specific organ physiological parameters as well as combining different types of FPBs within an MPS

(Table 1) will be explored to increase the ability to measure and to manipulate individual cells within the MPS. For example, monitoring one type of FPB in one cell type while activating another to modulate the cell physiology will permit real-time experimentation and testing.

Authors' notes: The materials and methods for the experimental data presented here are included in the supplementary material.

Authors' contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; NS, RB, LV, and RD conducted the experiments; NS, LV, RB, AG, and DLT wrote the manuscript. NS and LV have contributed equally to submitted work.

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