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Advances in measuring single-cell pharmacology *in vivo*

Claudio Vinegoni[1], J. Matthew Dubach[1], Greg M. Thurber[2], Miles A. Miller[1], Ralph Mazitschek[1] and Ralph Weissleder[1]

[1] Center for System Biology, Massachusetts General Hospital and Harvard Medical School, Richard B. Simches Research Center, 185 Cambridge Street, Boston, MA 02114, USA
[2] Department of Chemical Engineering, Department of Biomedical Engineering, University of Michigan, 2300 Hayward Avenue, Ann Arbor, MI 48109, USA

Measuring key pharmacokinetic and pharmacodynamic parameters *in vivo* at the single cell level is likely to enhance drug discovery and development. In this review, we summarize recent advances in this field and highlight current and future capabilities.

Introduction

Drug development often fails at late stages after significant cost and time investments [1]. With a current price tag of over US$1 billion per drug and an average of 13 years of investment [2–4], these figures are sobering. Furthermore, only three out of ten approved drugs manage to recover their respective development costs [5]. Therefore, the large upfront time and monetary expenditures limit the number of drugs that can be moved from the bench to the clinic. To accelerate drug development, and subsequently reduce exorbitant costs and high failure rates, the pharmaceutical industry needs to increase its overall R&D efficiency, not just productivity [1]. To this end, new tools are needed to study how drugs work *in vivo* and when and how they fail.

In the typical drug development process, small-molecule ligands are identified by screening diverse compound libraries against purified targets in biochemical assays. Hits derived from such screens are then further improved in an iterative medicinal chemistry process that includes profiling and validation in cell-based *in vitro* assays. This approach relies heavily on high-throughput screening methods, empirical and experimental compound selection and optimization, and the development of relevant animal models of disease. Additionally, computational techniques, including drug docking simulations and quantitative structure activity relationship (QSAR), are increasingly employed to improve the efficiency in lead compound optimization. Companies and research institutions have mastered this pipeline approach, often producing numerous promising drug candidates once a target has been validated. However, in the later stages of drug development, particularly preclinical animal testing and scaling to the clinic, quantitative mechanistic understanding at the molecular and cellular level is more difficult to attain. As such, the inability to determine drug behavior and downstream effects *in vivo* limits our understanding of drug pharmacology and is a major impediment to developing more-efficient medicines [1].

To enhance pharmaceutical development efficiency, optimized lead selection needs to be performed in conjunction with *in vivo* testing and analysis [1]. In particular, without methods to confirm that chemical probes reach and selectively engage their protein targets in living systems, it is difficult to attribute pharmacological effects to perturbation of the protein(s) of interest versus other mechanisms [6,7].

For a treatment to be successful *in vivo*, a drug must reach the cell of interest [pharmacokinetics (PK)] and engage with the molecular target, leading to a desired effect [pharmacodynamics (PD)]. Conventional pharmacology and PK/PD analyses measure this complex process at the organ or tissue level. Yet, what happens within populations of cells or at the single-cell level in real time often remains a mystery, particularly in the human setting where PK can be spatially distinct and cells exhibit heterogeneous phenotypes. Additionally, most drugs will bind to more than one target (polypharmacology), which can be favorable (inhibition of multiple kinases in cancer treatments) or unfavorable (side-effects) during systemic treatment. Therefore, measuring cellular pharmacology

Corresponding author: Vinegoni, C. (cvinegoni@mgh.harvard.edu)

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requires the use and development of new technologies, ideally those that can be applied *in vivo*.

Our laboratory has been at the forefront in the use and development of high-resolution intravital microscopy imaging for drug pharmacology through development of companion imaging drugs, fast imaging platforms, methods for automated processing, data analysis and machine learning, and perhaps equally important motion compensation. Together with advances in genetic reporters and mouse models, it is now possible to measure a multitude of cells and molecular events *in vivo* [8]. These include: (i) tissue, compartment and single-cell PK measurements; (ii) single-cell drug–target engagement measurements; and (iii) single-cell population PD measurements. Below we discuss current and new approaches to obtain these measurements with an emphasis on single-cell measurements *in vivo*.

Single-cell PK measurements

Traditional pharmacokinetics relies heavily on measurements of plasma concentration and is commonly described by the processes of absorption, distribution, metabolism, and excretion (ADME). Unfortunately, compartmental analyses do not consider potential tissue or cellular heterogeneity of drug distribution that can have significant effects on treatment response. Applications using fluorescent drugs in recent studies published from our group have provided direct, *in vivo* evidence of the presence of drug heterogeneity at the tissue [9], cellular [10] and subcellular levels [11], highlighting several routes of drug failure and potential solutions.

Following extravasation from blood vessels, small molecules diffuse and/or are carried by convection to the target cells. However, tumors have altered vasculature and elevated interstitial pressure reduces convection, which can result in large concentration gradients within the tissue. Therefore, decreased perfusion could produce insufficient drug distribution across the cellular population of the tumor. Owing to cellular genetic variability within tumors, cells exposed to suboptimal concentrations could then present a strong selection pressure that ultimately results in repopulation of the tumor by resistant clones. To follow drug distribution and uptake at the cellular level within a tumor, our group, and others, has developed fluorescently labeled drugs that maintain comparable specificity and affinity for the unlabeled drug target. When used in combination with confocal or two-photon fluorescence microscopy, fluorescent analogs provide high spatial and temporal resolution maps of drug distribution within the tissue, representing drug PK at the sub-organ or tissue level [9]. Furthermore, using detailed partial differential equation (PDE) modeling [11,12], the impact of different functional chemical groups and overall physiochemical properties can be determined. Predictive mechanistic models can then be compared to experimental results to help identify the role of various molecular parameters between the model and *in vivo* results [9], ultimately optimizing the underlying pharmacokinetic properties that drive homogeneous distribution (Fig. 1a).

Tumors are also composed of a heterogeneous mixture of cells with different genetic [13] and epigenetic [14] states, such as the presence of drug transporter proteins. Therefore, it is imperative to

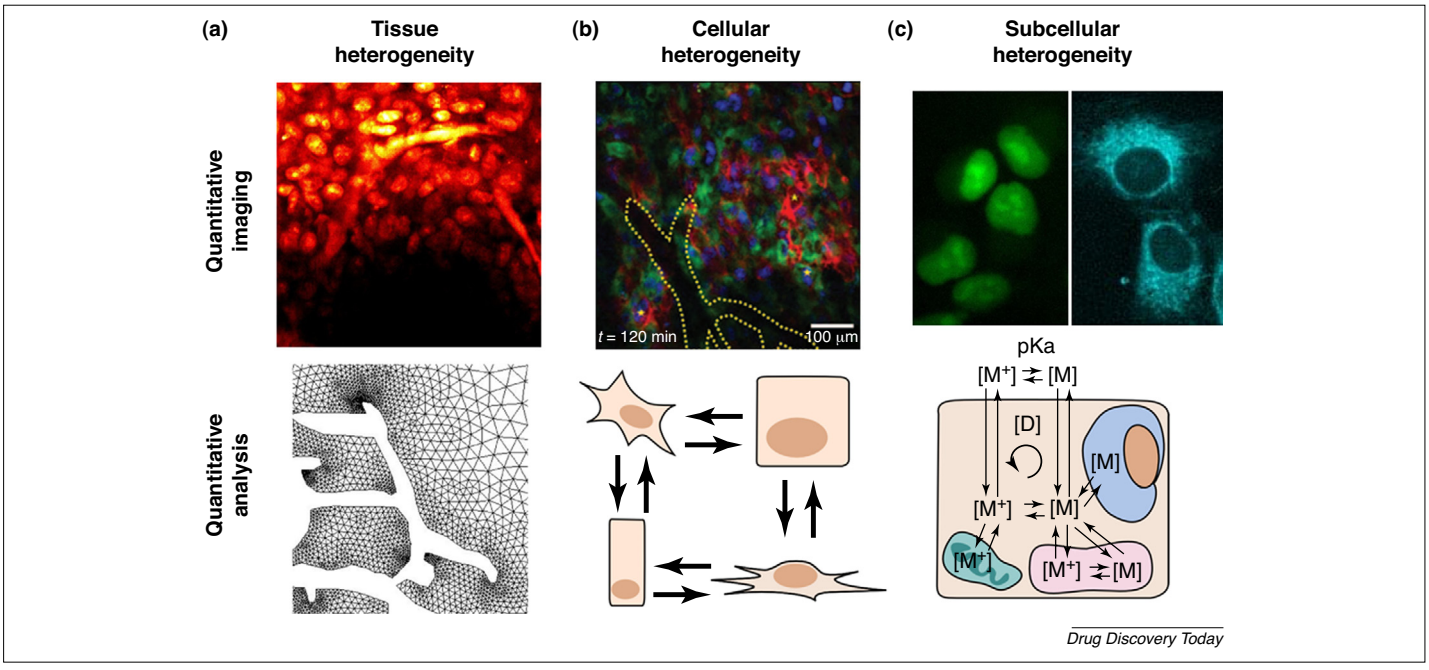

FIGURE 1

Single-cell pharmacokinetic (PK) measurements. The cellular location within tissue can determine the concentration time course of a drug. **(a)** Single cells can be tracked in relationship to vessels through intravital microscopy (top). For quantitative analysis, partial differential equation models using a finite element mesh based on the local vasculature (bottom) enable analysis of the distribution over time and extrapolation to other tumor microenvironments [8]. **(b)** Multichannel imaging can be used to identify different cell populations (endothelial cells, macrophages, fibroblasts, tumor cells) and clonal populations [such as P-glycoprotein 1 (PGP) expressing versus nonexpressing cells] (top) to measure uptake within these cells. Automated image analysis can be combined with the experimental data to quantify the variability in uptake and response (bottom) [9]. **(c)** Finally, drug properties can drive distribution into different subcellular compartments, which can be imaged under high magnification *in vivo* (top). Quantitative cellular models are compared to experimental results to determine if adequate concentrations are reaching the compartment of interest (bottom) [10].

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understand therapeutic agent distribution within each cell, particularly which available delivery methods result in the greatest cellular drug accumulation. Recently, we demonstrated that a fluorescent analog of eribulin, an inhibitor of microtubule dynamics, can be effectively used to study the activity and inhibition of P-glycoprotein 1 (PGP) during *in vivo* treatment of xenograft tumors [10]. Specifically, we found that cellular expression of PGP reduced accumulation of fluorescent drug, disrupting the cellular PK, yet this effect could be reversed by PGP inhibition (Fig. 1b).

Cellular resolution imaging of PK also enables differentiation of cell type within tissue. Several approaches to increase specificity of delivery to organs or tumors promise to decrease systemic toxicity of therapeutic treatment. For example, nanoparticle-based formulations can drive uptake of drug into tumor-associated macrophages, with drug subsequently reaching target cancer cells following nanoparticle release [15]. Furthermore, antibody drug conjugates preferentially deliver small-molecule drugs to cells expressing the target antigen. Therefore, measuring drug concentration in different cell types within tissue provides a resource to understand and optimize local or targeted delivery better.

The use of intravital confocal microscopy and fluorescent drug analogs also enables analysis of subcellular distribution *in vivo*. *In vitro* studies have indicated a correlation between physicochemical properties and partitioning into subcellular compartments [16–19]. Studies at the subcellular level, *in vitro* [20,21] and *in vivo* [11], can then be used to identify the interaction of the target-specific binding and nonspecific partitioning. This can be particularly relevant in the case of off-target activity in organelles (e.g. the mitochondria, endoplasmic reticulum) where concentrations can be higher than at the target of interest (Fig. 1c).

Single-cell drug–target engagement measurements

Ultimately, for a drug to be useful in the clinic, it must exert a pharmacological effect. The ability to detect target engagement of a drug with the cognate target can pinpoint to problems arising with intrinsic or acquired resistance due to target mutation. Recently a number of approaches have been developed to measure drug behavior in cells and *in vivo*.

The cellular thermal shift assay (CETSA) [22] has been used to measure engagement of unlabeled drugs with target proteins in cells and *in vivo*, with covalent drugs, as well as off-target binding of thousands of proteins within cells when combined with mass spectrometry [23]. Several other techniques have been recently developed including the use of positron emission tomography (PET) and mass spectrometry imaging (MSI) [24]. Unfortunately, the aforementioned techniques, although capable of determining target engagement, either lack the cellular resolution (PET) or the potential to make temporal measurements *in vivo* (MSI).

High spatiotemporal resolution determination of pharmacology and interaction with the target could provide valuable insight into the ever-adapting tumor setting. Cancer cells thrive in a dynamic and heterogeneous *in vivo* environment, constantly responding to surrounding cues including the drug therapy itself. Destructive sampling or *in vitro* measurements can miss the response or compensation to therapy, potentially losing out on opportunities for intervention, such as combination treatments. Therefore, techniques capable of measuring drug interaction *in vivo* at the cellular level would provide a window into these complex effects.

Recently, we applied fluorescence polarization to traditional intravital microscopy, for multiphoton fluorescence anisotropy microscopy (MFAM) to image, with high spatiotemporal resolution, the target engagement of fluorescent drug analogs [25]. Fluorescence polarization (FP), based on the excitation selectivity of polarized light, is an approach to measure drug–target interaction frequently used in *in vitro* assays. If polarized light is incident on an ensemble of randomly oriented fluorescent molecules, photoselection excites molecules aligned with the polarization of the light and emission will preferentially occur along the emission transition dipole moment. Therefore, the angular relation between excitation and emission determines the degree of anisotropy (r), as dictated by the molecular rotation correlation time, defined by the Stokes–Einstein equation [26]. Because this property is largely dependent on the size of the fluorescent molecule, when a small fluorescent molecule such as a small-molecule drug binds to a much larger protein the anisotropy is significantly increased. Also, because fluorescence anisotropy is an additive property, its measure provides the fraction-weighted sum of the two possible states (bound and unbound).

Extending FP to microscopy for drug–target engagement imaging can provide quantitative measurements with spatial and temporal information unavailable in traditional FP measurements or other approaches [27]. Analogously to what occurs in a one-photon process, two-photon photoselectivity can give rise to anisotropic fluorescence emission with an even greater degree of dependence making it particularly useful for measuring molecular orientations with a higher precision [26]. Our imaging system, which is based on two-photon absorption, will then provide in addition to the extended FP sensitivity all other characteristics that contribute in making multiphoton imaging advantageous for *in vivo* and *in vitro* imaging applications (i.e. extended penetration depth, high axial resolution, reduced scattering, low phototoxicity and high axial resolution). Through the use of our imaging system we have demonstrated we can follow the binding of a fluorescent analog of the poly(ADP ribose) polymerase (PARP) inhibitor olaparib to its target, and we have determined the degree of target engagement within a cell *in vitro* (Fig. 2a) [25]. This imaging approach is estimated to be capable of detecting concentrations well below 10 nmol and within volumes of approximately 1 femtoliter.

This approach also provided the temporal resolution necessary to follow the intracellular engagement rate of the drug analog, demonstrating the dynamics of target engagement within the subcellular (nuclear) target location (Fig. 2b). Furthermore, target engagement of the fluorescent analog was inhibited by competition with the unlabeled, clinical drug, indicating that the fluorescent analog is a valid model of olaparib, because it only binds to olaparib targets. Lastly, target engagement of the drug analog was measured in individual cells over time in xenograft tumors in mice following systemic drug delivery [25].

MFAM provides a method to validate intracellular drug–target engagement to determine the cellular heterogeneity of target engagement within a tumor, and to study the temporal aspects of engagement and dissociation from the target, all *in vivo*. This can potentially allow scientists to identify and understand

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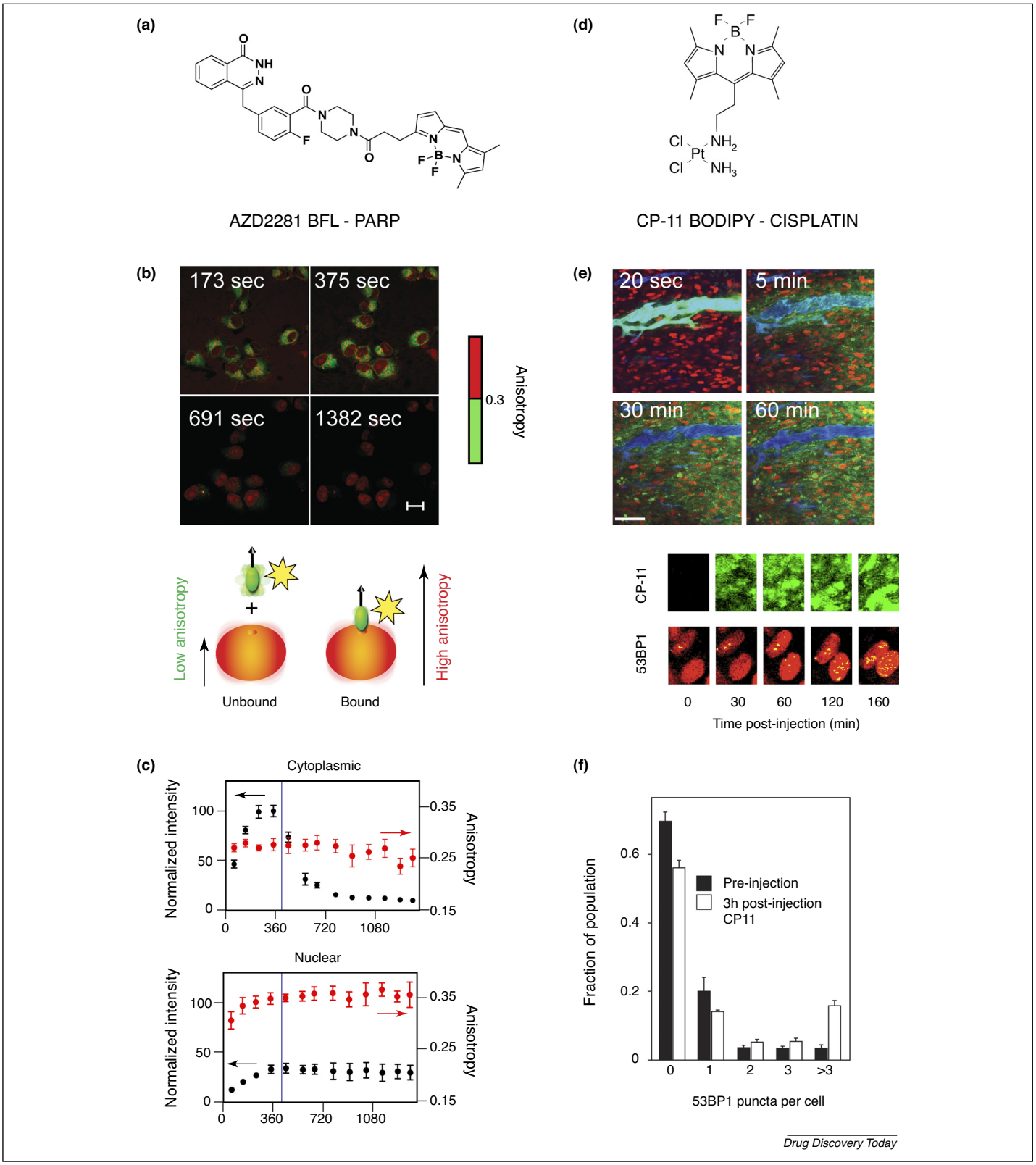

FIGURE 2

Single-cell drug target engagement and downstream pharmacodynamic (PD) measurements. **(a)** AZD2281 BFL-PARP molecular structure. **(b)** The fluorescence anisotropy value of a small-molecule fluorescent drug increases following binding to its own target, a much larger protein. Multiphoton fluorescence anisotropy microscopy (MFAM) provides high-resolution imaging of drug–target engagement within the subcellular target localization [24]. **(c)** Real-time imaging of engagement for HT1080 cells loaded with fluorescently labeled olaparib throughout the course of loading and washing (indicated by the blue line) [24]. **(d)** CP-11 Bodipy®–cisplatin molecular structure. **(e)** Bodipy® conjugated Pt-complexes can be used to image the PK and tumor uptake at the cellular level in a xenograft cancer mouse model. Following treatment with a fluorescent cisplatin derivative (CP-11), drug accumulation and downstream PD response via analysis of DNA damage response (DDR) foci formation within tumor cell nuclei can be monitored in real time at subcellular resolution *in vivo* [30]. **(f)** Cellular quantification indicates that CP-11 treatment causes a significant increase in the fraction of cells with more than three puncta per nucleus, at 3 hours post-injection [30].

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shortcomings of specific drug candidates earlier in preclinical development, and therefore avoid costly late-stage failure.

Single-cell downstream PD measurements

Drug–target binding initiates a series of subsequent changes in cellular pathways and behaviors, often in a highly context-dependent and heterogeneous manner across individual cells. Importantly, factors influencing such a downstream PD response can have tremendous impacts on drug efficacy, even in cases where the expression of the drug target itself, and corresponding drug binding to that target, remain unaffected [10,14,28]. Therefore, to correlate PK with successful treatment, it is essential to measure and understand not just PD as determined by drug–target engagement but downstream PD responses as well.

New technologies have recently been developed that enable measurements of downstream PD response at the single-cell level, with *in vivo* potential through intravital microscopy. As one strategy, fluorescent genetic reporters allow real-time measurement of various relevant cellular pathways, including mitogenic phosphosignaling activity, DNA damage response (DDR), cell cycle, calcium-channel signaling, metabolic state, apoptosis and protein expression [29,30]. Pathway dynamics can be crucial to understanding PD, and a key advantage of using fluorescent genetic reporters is the ability to perform longitudinal measurements. This is essential when connecting PK and target engagement to PD in *in vivo* models, because the time-course of drug distribution and target interaction can be significantly different from cellular response measurements, such as DDR. Advantageously, genetic reporters can be used in conjunction with fluorescent drug analogs and MFAM to provide an integrated PK/PD perspective *in vitro* and *in vivo*. For instance, recent work showed the ability to monitor the real-time distribution and single-cell uptake of a fluorescent derivative of the DNA-damaging chemotherapeutic cisplatin in a live-animal model of cancer [31]. Following treatment, simultaneous fluorescence imaging of DDR, via analysis of DDR foci formation within tumor cell nuclei, revealed highly heterogeneous DDR across individual tumor cells despite rapid and relatively uniform drug uptake and accumulation (Fig. 2c,d). These results reveal how, for certain therapeutics, the cellular response can be seemingly independent of cellular PK. Continued exploration into cellular signaling pathways, through genetic reporters, and target engagement dynamics could provide insight into why PD and PK do not correlate in this mode. Careful consideration of the experimental protocol should be considered with using genetic PD markers. Despite their advantages, genetic reporters are unfortunately limited in their applicability to certain cellular pathways and cell types, and simultaneous measurement of multiple reporters via multiplexed imaging is generally limited by spectral overlap of fluorescent proteins.

Concluding remarks

Overall, an integrated analysis that incorporates not just PK and the traditional PD of drug binding but also downstream PD responses is crucial for understanding rate-limiting barriers to drug efficacy *in vivo*, and mounting evidence has underscored the importance of single-cell heterogeneity in that equation. To this end, scientific progress is often driven by new tools that enable previously intractable measurements. Inspired by this paradigm, our team has built a toolbox to bring us closer to the long-cherished goal of making single-cell measurements *in vivo* in mouse models of human disease. At the onset we have had a particular interest in shedding light on the critical question: why do so many drugs fail? Using some of the tools summarized above we, and others, have made some surprising discoveries, found work-arounds, improved drug delivery and were able to test new targeting approaches. Although we have only scratched the surface, it has become clear that the developed tools are extraordinarily powerful. Although many of the above examples and first applications have focused on cancer, the imaging technology is now readily adaptable to other organs such as the heart, lungs and others.

What remains to be done and what are some of the limitations? There is a need for faster imaging allowing the deciphering of rapid biological processes (signaling, action potentials) or simply to cover larger imaging volumes per unit of time. The analyses of 3D volumes are not trivial and automated approaches remain to be developed to take full advantage of high-speed acquisitions. Otherwise, what good does a 10 min scan do if it takes 1 week to analyze the stack? A key aspect in all optical measurements is that we rely on fluorescent drug surrogates (the companion imaging drugs). These can behave differently from the parent drug and are only model systems. Although we have shown that they behave similarly in some targets, we have also encountered considerable difference in others. So, careful validation will always be necessary. Finally, the current measurements are still limited to mouse models because the repertoire of fluorescent drugs with human approval is limited. Incidentally, the development of human microscopy is not: there are intense efforts to bring microendoscopy and dermatological imaging to the mainstream. It is not hard to imagine the power these tools could have in conjugation with human companion imaging drugs.

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