ANNUAL REVIEWS

Annual Review of Pharmacology and Toxicology

Deciphering Drug Targets and Actions with Single-Cell and Spatial Resolution

Zhengyuan Pang,[1] Benjamin F. Cravatt,[2] and Li Ye[1,3]

[1]Department of Neuroscience, The Scripps Research Institute, La Jolla, California, USA; email: liye@scripps.edu

[2]Department of Chemistry, The Scripps Research Institute, La Jolla, California, USA; email: cravatt@scripps.edu

[3]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California, USA

Annu. Rev. Pharmacol. Toxicol. 2024. 64:507–26

First published as a Review in Advance on September 18, 2023

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

https://doi.org/10.1146/annurev-pharmtox-033123-123610

Keywords

drug-target interactions, mechanism of action, chemoproteomics, single cell, spatial omics, CATCH

Abstract

Recent advances in chemical, molecular, and genetic approaches have provided us with an unprecedented capacity to identify drug-target interactions across the whole proteome and genome. Meanwhile, rapid developments of single-cell and spatial omics technologies are revolutionizing our understanding of the molecular architecture of biological systems. However, a significant gap remains in how we align our understanding of drug actions, traditionally based on molecular affinities, with the in vivo cellular and spatial tissue heterogeneity revealed by these newer techniques. Here, we review state-of-the-art methods for profiling drug-target interactions and emerging multiomics tools to delineate the tissue heterogeneity at single-cell resolution. Highlighting the recent technical advances enabling high-resolution, multiplexable in situ small-molecule drug imaging (clearing-assisted tissue click chemistry, or CATCH), we foresee the integration of single-cell and spatial omics platforms, data, and concepts into the future framework of defining and understanding in vivo drug-target interactions and mechanisms of actions.

1. INTRODUCTION

Most drugs in clinical use are small-molecule compounds, often developed through screens to identify bioactive compounds that elicit a desired therapeutic effect. After candidates for development are nominated through such a route, a key downstream step is identifying the biomolecules (typically proteins) that interact with the compound. These so-called drug-target interactions (DTIs) provide information about the mechanism of action (MOA) of compounds, reveal additional molecular components in the drug signaling pathway, and uncover potential off-target effects. Historically, DTIs have largely been described at the molecular level, in terms of which biomolecule(s) engages with the drug (1–4). As our ability to directly assess changes in the levels of native biomolecule abundance at both the transcriptional and translational levels has expanded, our understanding of drug MOAs across genomics and proteomics has substantially increased.

In recent years, biology has begun to take another leap forward following the development of new technologies for cataloging and perturbing biomolecules at the level of single cells and with in-depth spatial resolution. Findings from the applications of these new tools to mammalian biology highlight the high degree of heterogeneity across cell types in different tissue compartments and even call into question the idea of a cell type (5). Single-cell and spatial omics methods that provide high-resolution intratissue localization of cell types and even subcellular localization of transcripts and proteins are revitalizing many areas of biology, including developmental biology, immunology, oncology, and neuroscience (6–8). These methods also present an enormous opportunity for pharmacology—integrating single-cell and spatial information into our understanding of drug interactions will dramatically accelerate drug development and inform rational drug design (9–11).

In this review, we briefly describe established methods for profiling DTIs as well as tracing in vivo drug distribution. We then discuss new opportunities and gaps in our existing knowledge revealed by single-cell and spatial omics techniques (**Figure 1**). Finally, we highlight emerging capacities that would allow us to understand drug actions in a spatially resolved, cell type– and molecular marker–defined context. Such additional information has the potential to guide the design of therapies with superior efficacy and reduced toxicity.

2. OVERVIEW OF ESTABLISHED METHODS TO IDENTIFY DTIs

Classic approaches for matching a bioactive compound with its target often relied on in vitro affinity methods (12, 13). Following the development of advanced mass spectroscopy and a collection of genomic/proteomic tools, a whole arsenal of technologies are now being deployed to decipher DTIs (14–17). We discuss here three broad classes of these tools: chemoproteomics, label-free target identification methods, and molecular biology profiling. Additional methods and further details about these tools and approaches are reviewed elsewhere in depth (18–20).

2.1. Chemoproteomic Strategies

Chemoproteomic approaches seek to identify differences in the proteome in cell or tissue extracts in response to the addition of the compound of interest (21). These approaches typically rely on mass spectrometry for proteome analysis, and as mass spectrometry methods have advanced, chemoproteomics has become a widespread, powerful approach to identify protein-drug interactions. Beyond simply screening for proteomic changes in the presence or absence of the drug (global chemoproteomics), there are sophisticated approaches that derivatize the drug compound in order to better capture interacting partners. In these approaches, the drug is used as the starting point to design a probe that contains (*a*) a recognition group that interacts with specific proteins or classes of proteins (e.g., kinases), (*b*) a reactive group that covalently

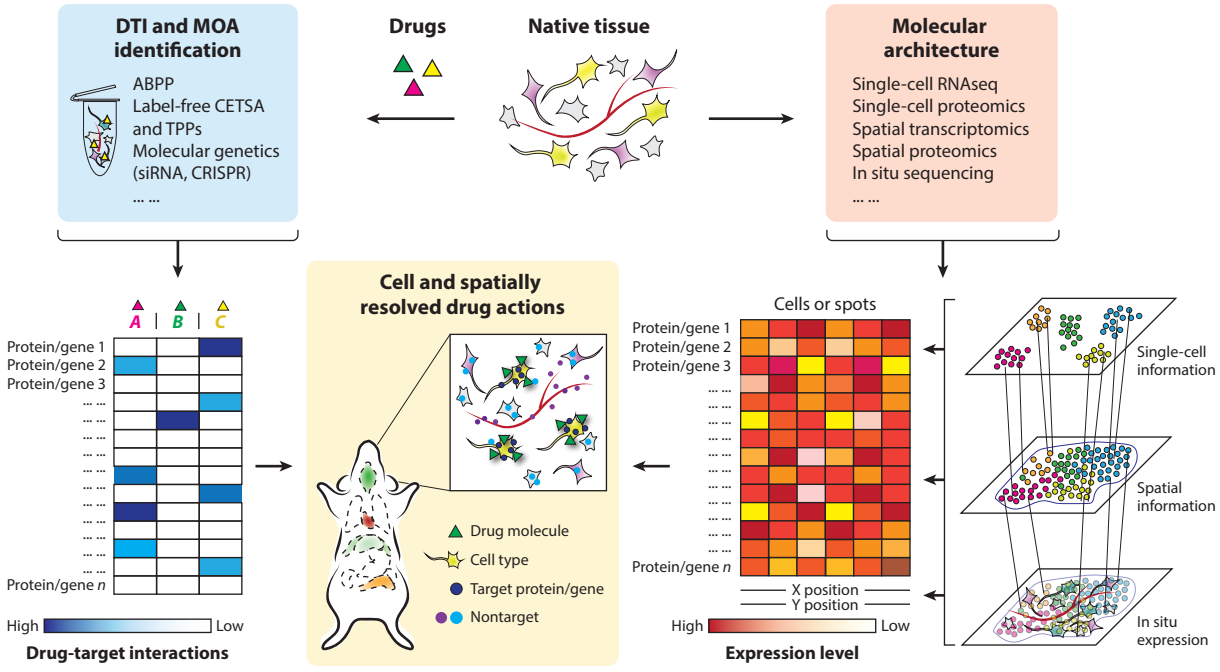

Figure 1

The gap between the conventional view of DTIs and the emerging heterogeneous architecture of mammalian tissues. Typically, targeting identification is based on drug-protein interactions in homogenized cell or tissue lysates with little cellular or spatial information, whereas newer technologies are rapidly revealing the importance of single-cell and spatial organizations in endogenous biological systems. Compared to the advanced methods for visualizing protein/DNA/RNA, there is a significant gap in how we visualize drugs and align them with spatially resolved molecule targets in vivo. Therefore, understanding in vivo drug actions with high cellular and spatial resolution remains an unmet challenge. Abbreviations: ABPP, activity-based protein profiling; CETSA, cellular thermal shift assay; CRISPR, clustered regularly interspaced short palindromic repeat; DTI, drug-target interaction; MOA, mechanism of action; RNAseq, RNA sequencing; siRNA, small interfering RNA; TPP, thermal proteome profiling.

captures target proteins through direct irreversible binding or photo-crosslinking, and (*c*) a reporter tag (i.e., biotin, fluorophore, and clickable alkyne/azide, etc.) that allows for the enrichment and identification of the target interacting proteins (15, 22). This type of approach is exemplified by activity-based protein profiling (ABPP), where activity-based probes (ABPs) are designed to read out the target proteins (22–24). ABPs can be obtained directly based on the compound of interest. Such a compound-centered strategy provides a global readout of drug binding targets. Upon ABP binding, the probe-target interaction can be stabilized via direct covalent bond formation or photo-crosslinking. Captured proteins can then be identified by gel or mass spectrometry analysis (**Figure 2*a***). This direct ABPP strategy has been successfully adopted to uncover the binding profiles of the neurotoxic compound BIA10-2474 (25, 26), the anti-inflammatory drug aspirin (27), and the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib (28). A variation on this approach, competitive ABPP, provides a conceptually simple yet efficient alternative (**Figure 2*a***). In competitive ABPP, the proteome is first treated with the drug of interest to occupy the probe binding site. A global ABP with broad proteome coverage is then applied to read out the competed targets. Initial efforts involving global ABPs were primarily associated with enzyme and kinase active sites (29–31) and have since expanded to reactive amino acid side chains (15, 31–35). Based on the ABP chosen, such a competitive approach provides the opportunity to profile drug binding

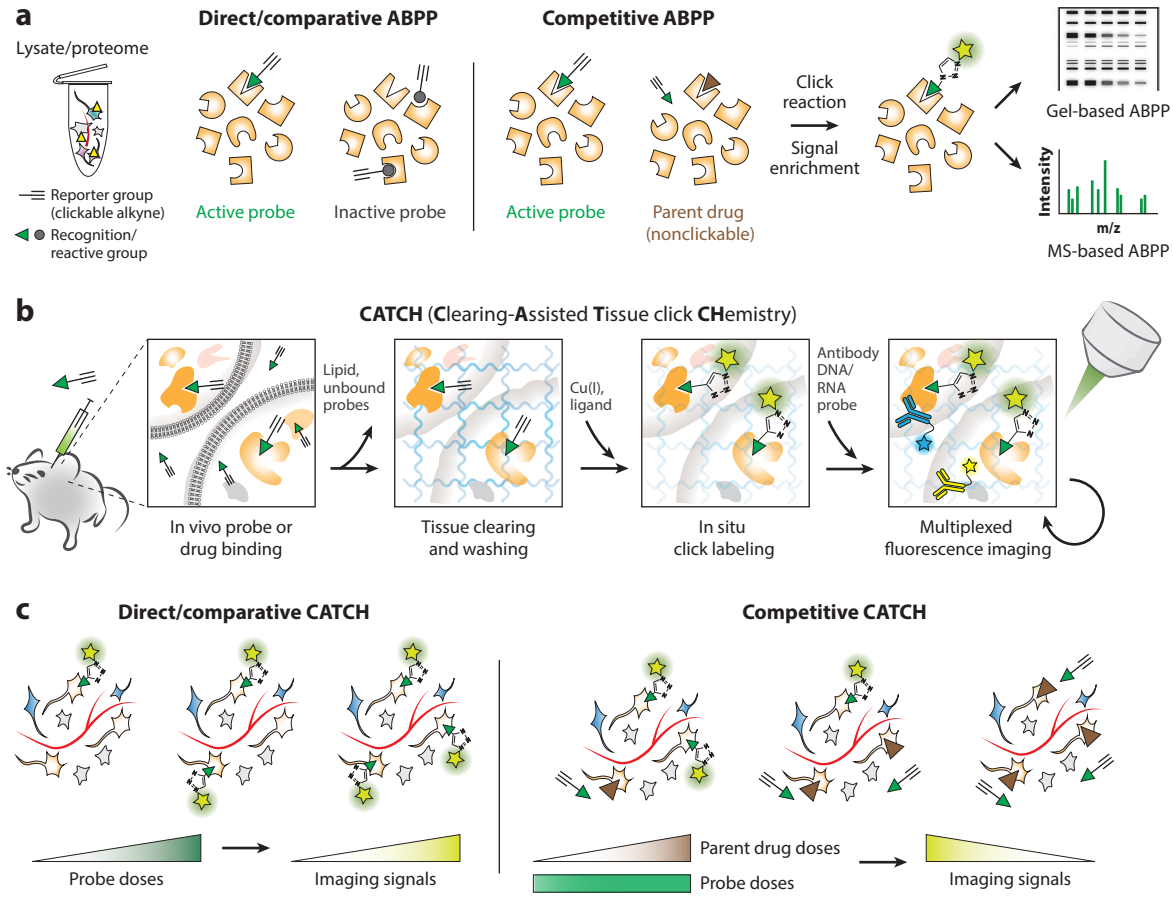

Figure 2

The general principles of ABPP and CATCH. (*a*) Schematics of a typical ABPP workflow. An ABPP probe consists of a recognition group guiding the probe to target molecules, a reactive group that forms a covalent bond between the probe and the molecular targets, and a reporter group (i.e., alkyne, biotin, or fluorophore) to profile probe binding. Cell culture or proteome lysates are treated with the ABPP probe and can undergo gel-based or MS analysis to profile molecular targets with probe binding. (*b*) A general workflow of in vivo CATCH using tissue clearing and click chemistry to fluorescently visualize target-bound drugs/probes in situ. The same ABPP probes in panel *a* can be directly adopted for CATCH. (*c*) Similar to ABPP, CATCH can be used in direct labeling and competitive configurations to quantitatively measure probe or parent drug binding. Abbreviations: ABPP, activity-based protein profiling; CATCH, clearing-assisted tissue click chemistry; MS, mass spectrometry.

proteins within a protein class. Finally, this strategy can be easily adopted to screen for selective ligands or peptide fragments that bind challenging proteins and even targets previously deemed undruggable (19, 36).

2.2. Label-Free Target Identification Methods

DTIs can also be measured based on changes to the biophysical properties of the interacting proteins. Based on the principle that ligands can induce protein stability changes, cellular thermal shift assays (CETSAs) were developed to monitor drug-target engagement (37). Initially, CETSAs relied on gel and western blot analysis for quantification (37). By combining CETSAs with quantitative mass spectrometry, thermal proteome profiling (TPP) was later developed for global DTI

profiling (38). The major advantage of TPP over most chemoproteomic strategies is that it does not require compound modification. TPP has been extensively employed to profile on- and off-target drug interactions in different cell cultures (39–42). More recently, TPP has been further developed to study DTIs in intact tissue and blood obtained from drug-treated animals (43). In addition to thermal stability, proteolytic and oxidative stability can also be used to measure drug-protein interactions (16). For a comprehensive discussion of the progress in and utilities of these strategies, we direct readers to reviews on these exciting technologies (see 4, 40, 44).

2.3. Profiling Methods Based on Molecular Genetics

These methods are based on loss-of-function or overexpression screens of endogenous proteins in the presence of a drug to infer MOAs. Early large-scale screening approaches relied on small interfering RNA (siRNA) libraries to identify genes that, when knocked down, altered the cellular response to a drug (45, 46). More recently, the RNA-guided CRISPR-Cas9 system has been developed to enable pooled genome-wide gene activation and repression screening (47–49). The integration of genomic perturbation and drug treatment can readily identify genes that are sensitive to drug treatment, thereby opening exciting opportunities for MOA elucidation and target deconvolution. For example, genome-wide CRISPR-Cas9 knockout screening was used to identify genes involved in resistance to the melanoma drug vemurafenib (47). Other advances in molecular biology profiling, such as the development of quantitative transcriptomics and proteomics tools, have provided the opportunity to globally investigate drug-induced changes at both transcriptional and translational levels. By screening multiple compounds and cross comparing the drug-induced changes and pathways involved, one can identify both messenger RNA (mRNA) and protein signatures associated with individual compounds, thereby inferring potential drug MOAs (17, 51, 52).

Collectively, the methods discussed above provide a rich toolbox for deciphering DTIs with high throughput, coverage, and resolution, especially in vitro. TPP and molecular profiling strategies have the advantage of not needing additional chemical modification of the candidate drugs and can provide a global view of DTIs. However, changes observed in the presence of the drug in these studies can stem from indirect mechanisms rather than direct drug-target binding. Affinity or activity-based chemoproteomic methods provide more direct evidence about drug-target binding and actions, but they generally require extensive work to design and validate the probe to ensure it maintains the identical activity of the original drug. One universal aspect of all these methods, however, is that they are most often conducted on bulk populations in either cell lysates or in cultured cells. Thus, these approaches are typically restricted to understanding DTIs in a homogenous, averaged, or diluted setting, which can obscure the identification and understanding of more complex interactions between a drug and its targets across different tissues and cell types.

3. CURRENT METHODS FOR STUDYING THE SPATIAL DISTRIBUTION OF DRUGS

It has long been recognized that the spatial distribution of drugs has a major impact on efficacy and toxicity. Insufficient drug exposure in target tissues and/or unwanted accumulation in other tissues can reduce potency and lead to potential side effects. Thus, in parallel to developing approaches to identify DTIs, there have also been efforts to capture spatial information about drug distribution. In a typical distribution analysis, blood is drawn, and individual organs are harvested and homogenized and then used to determine the drug concentration across the organ versus plasma. Organ lysate approaches, however, are low throughput, low resolution, and destructive. Alternatively, positron emission tomography (PET) can be used, which provides in vivo, real-time

measurements of drug distribution in the body (53). PET relies on radioisotope modification (such as ^{11}C or ^{17}F) to replace certain groups on the drug molecule to enable tracing. Because these substitutions minimally perturb the structure of the native drug, they typically have low or no impact on the original drug's activity. Currently, PET is widely utilized in preclinical studies and clinical diagnosis, and it is considered the gold standard for mapping drug distribution (54–57). However, the short half-life of the radioisotopes makes it technically challenging and expensive to synthesize the probe and perform imaging experiments in the limited time window before the radioisotope decays. It is also difficult to differentiate free versus target-bound drugs, complicating the interpretation of the results (58, 59). In terms of spatial resolution, PET can reveal differences at the level of the organ, and some intraorgan enrichment in larger organs, but it is difficult to observe cellular structure or differentiate single cells (59, 60). Finally, it is difficult to combine or multiplex PET-based imaging with mainstream cell identification methods, molecular markers, or other spatial omics methods, limiting the information that it can provide to relatively low depth and resolution.

4. CONCEPTUAL UPDATES ON TISSUE BIOLOGY AND PATHOLOGY THAT AFFECT THE UNDERSTANDING OF DRUG ACTIONS

We are now at an inflection point, once again transitioning to a new way of identifying DTIs in light of our updated understanding of tissue biology and pathology, which has come from applying new tools that can resolve differences at the level of single cells as well as capture spatial information (61–65). Over the past several years, single-cell and spatial omics data have provided a refined view of cellular function, leading to a reexamination of several assumptions about cell biology that are pertinent to understanding drug action, which we discuss below.

First, it used to be thought that one gene or protein could largely represent a specific cell type or even a specific organ (66), which led to the assumption that if a drug is specific to a certain highly expressed protein target, it would achieve specific cell type targeting. Although this concept is practical when bulk tissue or whole organs are analyzed and a list of top expressed genes is used for comparison (67), it has become clear that there is a substantial amount of heterogeneity in gene expression levels across all cells. In practical terms, this means that a tissue that appears to have low expression of a gene A in a bulk or averaged analysis could still contain cells or subtissue structures that express high levels of A. Thus, if a drug interacted with A, this type of localized off-target effect might not have been anticipated.

Second, it was previously assumed that one can use a so-called dominant cell type such as a hepatocyte or cardiomyocyte to represent the whole organ. This is the basis for classical in vitro models for studying drug efficacy and toxicity. We now recognize, however, the diverse roles that resident cells, blood vessels, immune cells, and neurons may have, despite being less abundant populations in an organ. Thus, the one cell type–one organ assumption needs to be revised. In a corollary to the above point, every tissue contains numerous molecularly diverse populations, and their engagement and interaction with drugs could have different implications.

Third, histology (i.e., imaging) has been a cornerstone for diagnosis and biomarker readout for treatment efficacy, whereas assaying molecular interactions (affinity/binding to protein) was mainly used for determining DTIs and drug MOAs. Historically, these two domains have been studied in isolation. However, with the advent of spatial omics, it is feasible to assay both in the same context (68, 69) and in the same sample and to causally link these two aspects. For example, we can now determine whether a certain histological feature, such as cell morphology, is associated with the expression of a particular gene, protein, or variant in that cell and capture information about the specific location of that cell within the organ or larger structure (70, 71).

The higher resolution afforded by single-cell and spatial omics tools is overcoming technical barriers and improving our understanding of cell function. There is thus an enormous potential to capitalize on these tools to rethink the basis of understanding drug actions and establish a new framework for studying them.

5. SINGLE-CELL AND SPATIAL OMICS TOOLS ARE TRANSFORMING BIOLOGY

The ideas discussed above are supported by studies using newly developed single-cell and spatial omics methods. These methods have the power to catalog molecular heterogeneity and diversity across tissues. More importantly, they provide quantitative experimental access to measure and manipulate such diversity and begin to directly associate molecular changes and drug-target engagement with dynamic histological and morphological architectures (72–74). It is fair to state that biology is rapidly entering a new era underpinned by spatially resolved omics data across different classes of biomolecules (DNA, RNA, proteins, metabolites), and it is critical that we consider drug interventions within this rapidly updating framework of general biology. Below, we provide a brief overview of some of these new tools and highlight their relevance to studying disease and developing drug treatments.

Perhaps the most widespread single-cell method is single-cell RNA sequencing (scRNAseq), which was developed over a decade ago (75, 76). scRNAseq provides in-depth transcriptional profiling across millions of cells at the scale of the whole genome and has revealed substantial levels of heterogenicity across all organs (7, 76). It has been applied to determine cell identity (77, 78); define transcriptional signatures (79); and assess gene expression changes in response to a perturbation or treatment, such as a drug response (80). One caveat of scRNAseq is that it loses spatial information as cells are dissociated prior to sequencing. There are also a number of extensions of scRNAseq such as Div-Seq, which assays transcripts in individual dividing cells (81); ATAC-Seq, which captures chromatin accessibility and transcript expression in the same cell (82); and Perturb-Seq, which integrates CRISPR screens with scRNAseq (83), to name just a few.

In parallel, and being amplified by scRNAseq techniques, spatial omics methods have been developed at an amazing speed. These methods can now resolve cellular, subcellular, and even single-molecule distributions of biomolecules in situ, allowing researchers to understand cellular functions and interactions within their native environments (73, 84). The most commonly used spatial omics methods include spatial transcriptomics (ST), which allows for the measurement of gene expression within individual cells or tissue regions (85), and spatial proteomics (SP), which enables the mapping of protein localization within tissues (86). Recent advances in spatial omics techniques have expanded their capabilities, including the development of spatial metabolomics and spatial lipidomics (87–89). Although these omics methods are quickly approaching untargeted, whole-genome/transcriptome/proteome-scale profiles (by in situ sequencing) of certain organs [e.g., mouse brain (90)], most practical applications of these methods rely on targeted probes based on scRNAseq analysis (corresponding to specific clusters or cell type markers). By combining several dozens of these probes, users can achieve spatial omics profiles of their tissue of interest.

Within the realm of drug development, we highlight here two key areas that exemplify the benefits of spatial omics methods: the tumor microenvironment (TME) and the central nervous system (CNS). Tumors are a complex tissue composed of tumor cells with varying genetic signatures; stromal cells, including fibroblasts, endothelial cells, and immune cells; and blood vasculature, and this heterogeneity is a critical challenge for effective cancer treatment (91). Early applications of scRNAseq provided the first quantitative measure of this heterogeneity (92–94). We have continued to expand our understanding of the TME with spatial omics techniques to uncover detailed molecular changes across this complex environment (95). For example, spatially

resolved metabolic modeling of prostate cancer TMEs revealed that fatty acid desaturase (SCD1) and prostaglandin transporter (SLCO2A1) are potential therapeutic targets (96). By combining ST and multiplexed ion beam imaging, Ji et al. (97) revealed a tumor-specific keratinocyte population critical for intercellular communication in squamous cell carcinoma. Based on these and many other recent findings (98, 99), it is clear that a detailed understanding of drug actions in tumor tissues at this new level of resolution can provide critical insights for evaluating drug efficacy and potential therapeutic resistance (100, 101).

The CNS is another highly complex organ orchestrated by various brain regions, cell types, and connections (102). Different ST techniques have been extensively employed to uncover the heterogeneity of the mammalian CNS (63–65, 103–105). More importantly, beyond advancing our understanding of circuit connections and regional/cell type functions, ST has become immensely valuable in uncovering mechanisms underlying neurological diseases. For example, ST revealed a plaque-induced gene network in tissues surrounding amyloid plaques, which was also observed in other neurodegenerative diseases (106). By combining ST and protein detection, Zeng et al. (107) found disease-associated microglia in close contact with amyloid plaques in a mouse model of Alzheimer's disease. The ever-expanding single-cell and spatial omics toolbox will allow us to characterize a wide range of, if not all, disease conditions with unparalleled resolution, generating information that will be highly relevant to drug discovery and optimization.

The molecular and spatial heterogeneity of biological systems revealed by these new methods suggest several scenarios that can benefit from resolving drug target engagement at a cellular level, mirroring the proposed concepts in Section 4. (*a*) Target gene/protein expression can vary across cells/cell types within the same organ. (*b*) Less abundant cells within the organ can have highly distinct expression patterns, information that is blunted by taking the average expression of the whole organ, yet these cells could have disproportionally large biological roles. (*c*) The same protein target can have different intracellular signaling in different cell types depending on the expression of downstream pathway components or scaffold/partner proteins. (*d*) Even for protein targets in the same cell type, with the same downstream signaling pathways, the result of a drug interaction with this target can still result in a different physiological or pathological response depending on where the cell is (e.g., hitting a protein target on a T cell in the spleen versus a T cell in the gut could have different consequences; similarly, targeting GABAergic neurons can inhibit different parts of the brain leading to distinct behavioral outcomes).

Examples of such relevant heterogeneity are already documented in the brain and TME, and it is reasonable to expect that it will be found in other organs and tissues as ST and SP technologies are quickly being adopted to reveal the molecular and morphological details of native tissue architecture (108–110). Given these rapid advances in understanding cellular function in healthy and diseased states, it is imperative to develop matching abilities to map drug actions on this new landscape of tissue architecture.

6. VISUALIZING TARGET-BOUND DRUG MOLECULES IN SITU

As we move toward having both an omics- and in situ spatial-level understanding of native biology, it is important to understand how drug perturbations affect tissues at the same scale and resolution. One current approach is to use cellular and spatial gene expression changes to measure drug response and infer the MOAs. For example, immunostaining and histology of immediate early genes (like *cFos*) are commonly used to assay the cellular response to certain drugs in the CNS (111–114). Although these methods have sufficient resolution to characterize cellular and spatial heterogeneity, they are indirect and rely on the performance of the biomarker to report the actions of the drug. This may or may not be the primary response or even a direct response

(e.g., biomarker activity can be confounded by other compensatory cells or signaling mechanisms or subject to the temporal rebound of the primary drug action).

Ideally, a more precise way to achieve high resolution of drug activity would be to directly observe the primary binding site of the drug molecules. With recent advances in optical imaging resolution, which can now achieve single-molecule resolution for DNA and proteins (115–117), it should now be possible to image target-bound drug molecules in situ at their primary binding sites. Such an ability would enable researchers to register binding site with cellular identification, organization, morphology, tissue microenvironment, and so on, as well as with gene expression data in situ to directly characterize drug MOAs.

Fluorescence imaging forms the foundation for imaging-based spatial omics methods. This is done by adding a fluorescent tag to an endogenous protein or mRNA (via genetic encoding) and to an affinity probe (antibody or oligo) targeting endogenous molecules. There are two major barriers to employing this strategy for small-molecule drugs. First, the relatively large size of the fluorescent tag (hundreds to thousands of daltons) compared to the size of a typical drug molecule (<500 Da) means that the tag is very likely to affect the affinity, stability, and ultimately in vivo biological activity of the drug. Thus, it is hard to use a fluorophore-tagged drug as a surrogate for the parent drug, especially for in vivo DTI discovery. Second, the tag added to the drug needs to be inert in the biological system under study (both in vivo and during the imaging process) to ensure it does not react with native molecules or cause endogenous molecules to confound the imaging readout (this is the key idea of the concept of bioorthogonality) (118). Nevertheless, this approach has been used, notably, to couple fluorophore-tagged drugs with stochastic optical reconstruction microscopy (STORM) in a method called PharmacoSTORM (119). However, it can be challenging to ensure the fluorescence probe equivalently captures the in vivo behaviors and actions of the parent drugs.

The above two barriers are not new or unique to imaging applications; during the development of ABPP/chemoproteomics, bioorthogonal click reactions were adapted specifically to address these problems (15, 120). Instead of a bulky fluorophore tag, ABPs introduce a small click handle (alkyne, azide, etc.) to minimize the impact on native drug activity. The addition of the fluorophore tag only happens after the in vivo target-drug binding occurs and is stabilized either by a covalent mechanism or by additional crosslinking, thereby enabling readout. This click-ABP strategy is well established and offers several unique advantages: (*a*) minimal changes to the properties of the drug; (*b*) high versatility compatible with imaging, gel, or mass spectrometry analysis as different functional additions can be added afterward to the target-bound drug, and (*c*) the doses and sequence of parent drug–click probe combinations can be easily modified to acquire quantitative binding information through competitive binding assays (**Figure 2*a***). As summarized above, these methods have been widely and successfully used to identify DTIs in cell lysates.

Several attempts have been made to adopt chemoproteomic probes for fluorescence imaging, for example, in cell culture systems, flow cytometry applications, and in vivo live imaging (121–125). These pioneering works provided powerful tools for studying protease activities (124, 126, 128, 129) and hormone (130) and kinase signaling (28, 131, 132), as well as for guiding tumor margins during surgery (126, 133, 134). However, it is not clear whether the resolution and multiplexing compatibility of these methods can match that of spatial omics imaging approaches in tissues. A major challenge is that the signal-to-noise ratio of the click reaction in dense mammalian tissue is relatively low.

In a recent report by Pang et al. (135), we identified that a key enabling step for allowing highly efficient, in situ tissue click chemistry is to delipidate the tissues before the reaction, which can be achieved by a series of recently developed tissue-clearing techniques [such as CLARITY—readers are referred to other reviews on the topic (136, 137)]. By integrating delipidation and

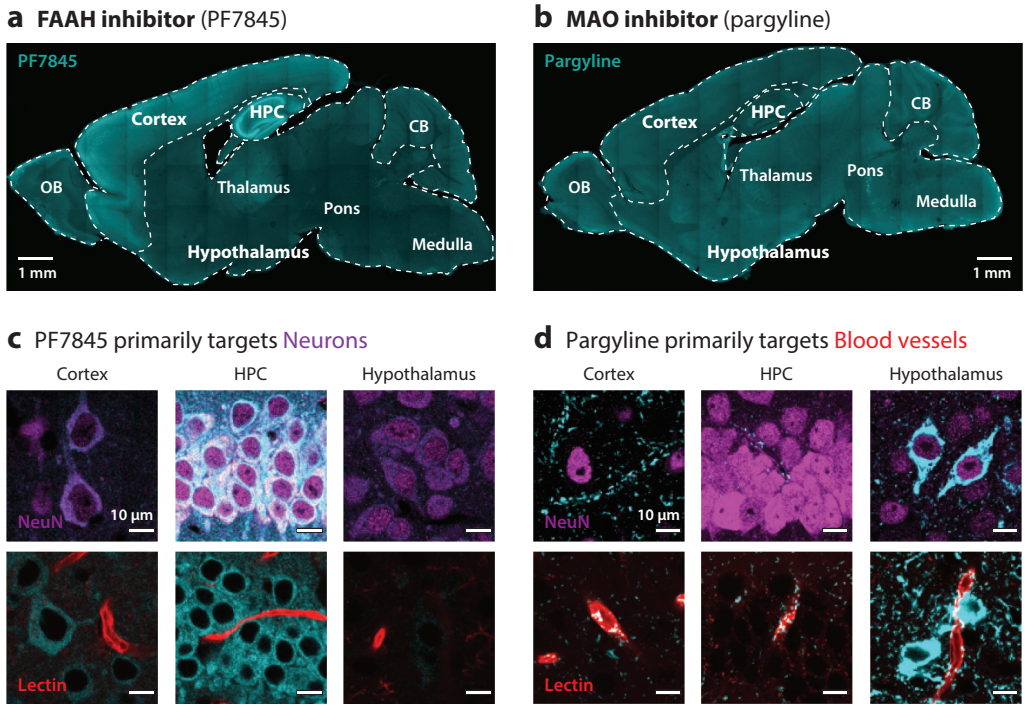

Figure 3

CATCH for region/cell type–specific drug binding identifications. (*a*,*b*) CATCH allows for brain-wide drug binding profiling. Images show FAAH inhibitor PF7845 (*a*) and MAO inhibitor pargyline (*b*) binding. (*c*,*d*) CATCH is compatible with additional marker staining for cell type characterizations. Images show zoomed-in views with NeuN antibody staining for neuron and lectin staining for blood vessels. (*c*) PF7845-yne binds to neurons but not blood vessels. (*d*) Pargyline-yne shows pervasive blood vessel binding and strong neuronal engagement in the hypothalamus. Abbreviations: CATCH, clearing-assisted tissue click chemistry; CB, cerebellum; FAAH, fatty acid amide hydrolase; HPC, hippocampus; MAO, monoamine oxidase; NeuN, neuronal nuclei; OB, olfactory bulb. Figures adapted from Reference 135.

ligand optimization procedures prior to click reactions, we developed clearing-assisted tissue click chemistry (CATCH) (**Figure 2*b*,*c***). This allowed us to visualize covalent drug binding at subcellular resolution in situ in mouse brain tissues (**Figure 3*a*,*b***). Because CATCH is compatible with mainstream histological methods such as immunostaining and in situ hybridization (**Figure 3*c*,*d***), it can identify where and which cell types within an organ are quantitatively bound by a specific drug, revealing heterogeneous intraorgan drug engagement.

We applied CATCH to investigate the spatial behaviors of multiple drugs in the CNS. We found that PF7845 and BIA10-2474, two fatty acid amide hydrolase (FAAH) inhibitors, primarily target neurons in the brain, whereas pargyline, a monoamine oxidase inhibitor, mostly binds blood vessels. In agreement with previous findings that another FAAH inhibitor, BIA10-2474, is less specific (26), we observed that BIA10-2474 exhibited off-target binding in a small nucleus in the pons. Finally, we saw that as the dose of the monoacylglycerol lipase inhibitor MJN110 increased, it spread from the axonal to the soma compartment through off-target binding to the FAAH.

In addition to revealing drug binding across cell types in a dose-dependent manner, CATCH has two important advantages. First, CATCH directly utilizes and is natively compatible with any ABPP click probes. This not only allows for use of ABPP proteomic data to infer and interpret CATCH imaging results, linking molecular and cellular affinity through the shared

probes, but also, and more importantly, allows both direct CATCH and competitive CATCH to be used for dose-dependent quantification of DTI, mirroring the comparative ABPP versus competitive ABPP complementation (**Figure 2*c***). Such a two-way interrogation ensures the fidelity of target profiling and improves its quantitative capabilities. Second, because CATCH is rooted in polymer-based volumetric tissue clearing and imaging, it is natively compatible with multiplexed molecular characterization (spatial omics ready)—for example, we demonstrated that immunostaining reagents (used for cell type identification) can be washed away, and the tissue can be restained with several rounds of additional marker stains. It is conceivable that CATCH can be extended and combined with sequential labeling/multiplex/in situ sequencing methods such as spatially resolved transcript amplicon readout mapping (STARmap) (138) (which also shares its roots with CLARITY). Through these extensions, CATCH could serve as a bridge to link molecular-level omics drug affinity to tissue-level, cellular-resolution spatial omics imaging data.

7. LIMITATIONS OF CURRENT CATCH METHODOLOGY AND FUTURE DEVELOPMENTS

We have demonstrated CATCH applications primarily for covalent, irreversible drugs (i.e., ones that form covalent bonds with their targets). Throughout the history of pharmaceutical development, covalent drugs, including, for instance, aspirin, penicillin, and proton pump inhibitors, have proven to be some of the most transformative drugs in medicine. Despite their proven success and inherent advantages, however, there has been a general reluctance to develop covalent drugs due to the concern of potential off-target toxicity across different organ systems (139). Hence, a comprehensive understanding of in vivo targets is particularly critical for this potent class of drugs.

Recently, however, there has been renewed interest in developing covalent drugs to achieve major goals in the drug discovery industry for targets that have proven elusive for more classical, reversible small molecules, including, for instance, the selective inactivation of oncogenic and autoimmune kinases [BTK, epidermal growth factor receptor (EGFR), fibroblast growth factor receptors (FGFRs), Janus kinase 3 (JAK3)] and, most notably, the inhibition of the once-deemed undruggable Kirsten rat sarcoma virus (KRAS) protein (140, 141). The relevance of covalent drugs also extends to infectious disease targets, as reflected in the antiviral protease inhibitors serving as breakthrough therapies for hepatitis C (142) and SARS-CoV-2 infections (143) (e.g., sofosbuvir and Paxlovid). Despite this resurgence of interest in covalent drugs across therapeutic areas, including oncology, immunology, neuroscience, and infectious disease, improving their efficacy while minimizing toxicity by optimizing tissue selectivity, especially for the CNS, remains challenging. CATCH has the potential to greatly accelerate this process by revealing the drug's cellular engagement in both on-target and off-target organs, thereby guiding lead selection and downstream modifications.

A key remaining goal is to further develop CATCH for reversible drugs, as most current drugs are not covalent. Recently, Nonaka et al. (144) developed a strategy termed fixation-driven chemical cross-linking of exogenous ligands (FixEL), whereby a drug is modified with an amine group, which allows the drug to be cross-linked with tissue proteins during formaldehyde fixation. However, as the primary amine can potentially react with endogenous proteins in proximity, it remains to be determined how this linker may affect the drug activity (i.e., whether the amine-drug probe can be considered as an equivalent of the parent drug, especially in vivo). A more common strategy used in chemoproteomics approaches is to install a photoactivatable cross-linker (most commonly diazirine) so that the cross-linking can only happen under additional activation, such as with ultraviolet (UV) light (145). It is conceivable that this strategy could be adapted to design CATCH probes for reversible drugs, taking advantage of the rich literature and established technology on

diazirine-based chemoproteomics. However, high-energy light usually does not penetrate deep tissues well in vivo (it is limited to a few millimeters), thus this strategy is likely to have relatively restricted tissue coverage in the case of whole tissues or low throughput in the case that the tissue is sectioned before UV activation. One potential workaround to this limitation is to explore other chemical or physical catalyst-mediated cross-linkers that may be substituted for photoactivatable ones.

8. OUTLOOK: CATCH TO BRIDGE PHARMACOLOGY AND SPATIAL OMICS

Based on the success to date, we foresee CATCH or other in situ high-resolution drug-imaging methods contributing greatly to our understanding of DTIs and drug MOAs in the single-cell and spatial omics era. Critically, CATCH has the ability to bridge traditional proteomic-level molecular affinity information (1D omics data) with cellular-resolution spatial omics expression (2D/3D omics) data and direct, in situ, information from the primary site of action.

Traditional DTI methods can reveal which protein(s) binds to a drug or whether the expression of any protein(s) or biomarker(s) is altered by a drug, but they cannot tell where and in which host cell type these responses occurred. As discussed above, variation in host cell types or their spatial niches can significantly alter the biological outcomes and interpretations of DTI experiments. Early in drug development, initial DTIs are still frequently based on lysate-based profiling (such as affinity/activity/bio response–based methods, reviewed above). At later stages, CATCH can be utilized to identify the primary host cell types to determine whether the binding/intervention was on the intended cellular or subtissue structure. Such CATCH profiling can be done in individual tissues based on their biological relevance, or it can potentially be scaled up to whole-body unbiased screens (in rodents), as single-cell resolution, intact whole-mouse clearing and light-sheet imaging have been shown to be feasible (146–148).

The identification of cellular targets (and additional in situ molecular characterization, up to the spatial omics level) can be particularly useful in prioritizing multiple lead compounds based on which one has the most enriched binding in the relevant cell types (or the fewest off targets in other cell types; see below). Moreover, we envision that the cellular target could be incorporated into the structure-activity relationship readout to select for lead compounds with better enrichment in the on-target cell types to optimize drug candidate selection. For example, brain-penetrating BTK inhibitors (such as evobrutinib and tolebrutinib) are showing positive results in treating multiple sclerosis (MS) in human trials (149, 150). However, little is known about their in vivo CNS targets. Whether the primary targets are B cells and myeloid cells, microglia, or oligodendrocytes in the brain is still under debate (151, 152). Identifying the cellular and anatomical targets of BTK inhibitors in vivo would greatly accelerate our understanding of MS. For example, one can specifically design additional in vitro cell type–specific (based on CATCH findings) screens to identify more effective and selective BTK inhibitors for optimizing BTK inhibitor–based MS treatment.

Beyond initial drug discovery, CATCH can also be used to help guide precision treatment strategies for heterogeneous cancers. For example, it was recently reported that an acquired human epidermal growth factor receptor 2 (HER2) T798I gatekeeper mutation induced resistance to neratinib in a patient with HER2 mutant–driven breast cancer (153). In vitro models suggest this T798I mutant is sensitive to the irreversible EGFR inhibitor osimertinib (153). CATCH could be used in vivo, together with nucleic acid probes, to determine whether osimertinib binds to those cells with the T798I mutants within the heterogenous tumor tissues, providing insight into the potential benefits of a combination strategy using neratinib and osimertinib.

Conversely, the information revealed by CATCH can be used to identify and to filter out those drug compounds that have higher enrichment in the off-target cell types that may be associated with toxicity. For example, we found that the FAAH inhibitor BIA10-2474 has FAAH-independent but enriched binding in the pons, which may be related to its neurological toxicity (135, 154). CATCH could also be applied to improve existing treatments. For example, the covalent BTK inhibitor ibrutinib has revolutionized the treatment and prognosis of B cell malignancies (155), but it is associated with significant cardiovascular toxicity (156). Because such toxicity could result from and vary across different cardiac cells (cardiomyocytes, conduction system) and noncardiac cells (immune cells, endothelial cells, fibroblasts) (157, 158), it has been difficult to identify the underpinning primary cell type target for the drug-associated complications. Previously, chemoproteomics work showed that C-terminal Src kinase (CSK) is a key off target of ibrutinib, an interaction that has been associated with atrial fibrillation (159). However, it is unclear which cellular target is responsible for this off target and its associated toxicity (and in which cells). CATCH could potentially identify the main binding site of ibrutinib in the cardiovascular system, and additional ABPP studies could pinpoint the responsible molecular interactions leading to the toxicity.

In the near future, this reciprocal use of CATCH and ABPP (or other DTI methods) may be the most effective strategy to guide drug development and refinement. CATCH can initially be deployed to guide the selection of relevant tissues and cell types for in vitro screening, narrowing down the search space. Then, ABPP or other chemoproteomics methods that currently have much higher resolution, coverage, and depth across the proteome can be used to identify molecular targets. The next round of drug candidate screening (either in vitro or in silico) would select for or against these protein targets to enhance the desired cellular target and minimize the undesired off targets, and the results of this screen would subsequently be validated by a second round of in vivo CATCH. Additional iterations of this cycle could potentially further maximize on-target selectively while abrogating any off-target effects. A combined approach like this will take full advantage of the rich and complex biology revealed by the recent application of single-cell and spatial omics tools and begin to integrate drug-induced perturbations into a multimodal, multidimensional biological framework.

Ultimately, if in situ omics methods fully advance to the depth and coverage of lysate-based sequencing and mass spectroscopy approaches, in situ proteomic-scale ABPP/chemoproteomic methods could potentially provide all-in-one resolution to identify tissue structure, cell morphology, cell type, and molecular affinity information in one unified platform without going back and forth between in vivo and in vitro screens. As in situ multiplex imaging methods are rapidly developing, given the high degree of native compatibility between CATCH and multiplex tissue imaging as well as between CATCH and ABPP, we envision that future parallel development of CATCH will play a key role in enabling this unified platform. Using such a platform will bring our understanding of in vivo DTIs and drug MOAs into alignment with the emerging framework of single-cell, spatially resolved biological systems, accelerating the development of safe, efficacious therapies.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We would like to thank all members of the Ye lab and Cravatt lab for their support and feedback. Z.P. is supported by the Dorris Scholar Award. L.Y. acknowledges the support from the National

Institutes of Health Director's New Innovator Award (DP2DK128800), NIDDK (DK114165, DK134609, and DK124731), BRAIN initiative/NIHM (MH132570), NIDA (DA059393), NIC (CA281918), the Dana Foundation, the Whitehall Foundation, the Baxter Foundation, and the Abide-Vividion Endowment. B.F.C. acknowledges financial support from the NIH (DA033760 and CA231991).

LITERATURE CITED

1. Vamathevan J, Clark D, Czodrowski P, Dunham I, Ferran E, et al. 2019. Applications of machine learning in drug discovery and development. *Nat. Rev. Drug Discov.* 18:463–77
2. Finan C, Gaulton A, Kruger FA, Lumbers RT, Shah T, et al. 2017. The druggable genome and support for target identification and validation in drug development. *Sci. Transl. Med.* 9:eaag1166
3. Davis RL. 2020. Mechanism of action and target identification: a matter of timing in drug discovery. *iScience* 23:101487
4. Mateus A, Kurzawa N, Perrin J, Bergamini G, Savitski MM. 2022. Drug target identification in tissues by thermal proteome profiling. *Annu. Rev. Pharmacol. Toxicol.* 62:465–82
5. Zeng H. 2022. What is a cell type and how to define it? *Cell* 185:2739–55
6. Moses L, Pachter L. 2022. Museum of spatial transcriptomics. *Nat. Methods* 19:534–46
7. Elmentaite R, Dominguez Conde C, Yang L, Teichmann SA. 2022. Single-cell atlases: shared and tissue-specific cell types across human organs. *Nat. Rev. Genet.* 23:395–410
8. Stuart T, Satija R. 2019. Integrative single-cell analysis. *Nat. Rev. Genet.* 20:257–72
9. Srivatsan SR, McFaline-Figueroa JL, Ramani V, Saunders L, Cao J, et al. 2020. Massively multiplex chemical transcriptomics at single-cell resolution. *Science* 367:45–51
10. Wu Z, Lawrence PJ, Ma A, Zhu J, Xu D, Ma Q. 2020. Single-cell techniques and deep learning in predicting drug response. *Trends Pharmacol. Sci.* 41:1050–65
11. Vinegoni C, Dubach JM, Thurber GM, Miller MA, Mazitschek R, Weissleder R. 2015. Advances in measuring single-cell pharmacology in vivo. *Drug. Discov. Today* 20:1087–92
12. Zheng W, Li G, Li X. 2015. Affinity purification in target identification: the specificity challenge. *Arch. Pharmacal Res.* 38:1661–85
13. Kawatani M, Osada H. 2014. Affinity-based target identification for bioactive small molecules. *MedChemComm* 5:277–87
14. Meissner F, Geddes-McAlister J, Mann M, Bantscheff M. 2022. The emerging role of mass spectrometry-based proteomics in drug discovery. *Nat. Rev. Drug. Discov.* 21:637–54
15. Parker CG, Pratt MR. 2020. Click chemistry in proteomic investigations. *Cell* 180:605–32
16. Ha J, Park H, Park J, Park SB. 2021. Recent advances in identifying protein targets in drug discovery. *Cell Chem. Biol.* 28:394–423
17. Fellmann C, Gowen BG, Lin PC, Doudna JA, Corn JE. 2017. Cornerstones of CRISPR-Cas in drug discovery and therapy. *Nat. Rev. Drug. Discov.* 16:89–100
18. Vu V, Szewczyk MM, Nie DY, Arrowsmith CH, Barsyte-Lovejoy D. 2022. Validating small molecule chemical probes for biological discovery. *Annu. Rev. Biochem.* 91:61–87
19. Spradlin JN, Zhang E, Nomura DK. 2021. Reimagining druggability using chemoproteomic platforms. *Acc. Chem. Res.* 54:1801–13
20. Vincent F, Nueda A, Lee J, Schenone M, Prunotto M, Mercola M. 2022. Phenotypic drug discovery: recent successes, lessons learned and new directions. *Nat. Rev. Drug. Discov.* 21:899–914
21. Bantscheff M, Drewes G. 2012. Chemoproteomic approaches to drug target identification and drug profiling. *Bioorg. Med. Chem.* 20:1973–78
22. Niphakis MJ, Cravatt BF. 2014. Enzyme inhibitor discovery by activity-based protein profiling. *Annu. Rev. Biochem.* 83:341–77
23. Racioppo B, Qiu N, Adibekian A. 2023. Serine hydrolase activity-based probes for use in chemical proteomics. *Isr. J. Chem.* 63:e202300016
24. Cravatt BF. 2023. Activity-based protein profiling – finding general solutions to specific problems. *Isr. J. Chem.* 63:e202300029

25. Huang Z, Ogasawara D, Seneviratne UI, Cognetta AB 3rd, am Ende CW, et al. 2019. Global portrait of protein targets of metabolites of the neurotoxic compound BIA 10-2474. *ACS Chem. Biol.* 14:192–97
26. van Esbroeck ACM, Janssen APA, Cognetta AB 3rd, Ogasawara D, Shpak G, et al. 2017. Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474. *Science* 356:1084–47
27. Bateman LA, Zaro BW, Miller SM, Pratt MR. 2013. An alkyne-aspirin chemical reporter for the detection of aspirin-dependent protein modification in living cells. *J. Am. Chem. Soc.* 135:14568–73
28. Lanning BR, Whitby LR, Dix MM, Douhan J, Gilbert AM, et al. 2014. A road map to evaluate the proteome-wide selectivity of covalent kinase inhibitors. *Nat. Chem. Biol.* 10:760–67
29. Patricelli MP, Giang DK, Stamp LM, Burbaum JJ. 2001. Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. *Proteomics* 1:1067–71
30. Patricelli MP, Szardenings AK, Liyanage M, Nomanbhoy TK, Wu M, et al. 2007. Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* 46:350–58
31. Cravatt BF, Wright AT, Kozarich JW. 2008. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.* 77:383–414
32. Hahm HS, Toroitich EK, Borne AL, Brulet JW, Libby AH, et al. 2020. Global targeting of functional tyrosines using sulfur-triazole exchange chemistry. *Nat. Chem. Biol.* 16:150–59
33. Ma N, Hu J, Zhang ZM, Liu W, Huang M, et al. 2020. 2*H*-Azirine-based reagents for chemoselective bioconjugation at carboxyl residues inside live cells. *J. Am. Chem. Soc.* 142:6051–59
34. Abbasov ME, Kavanagh ME, Ichu TA, Lazear MR, Tao Y, et al. 2021. A proteome-wide atlas of lysine-reactive chemistry. *Nat. Chem.* 13:1081–92
35. Kuljanin M, Mitchell DC, Schweppe DK, Gikandi AS, Nusinow DP, et al. 2021. Reimagining high-throughput profiling of reactive cysteines for cell-based screening of large electrophile libraries. *Nat. Biotechnol.* 39:630–41
36. Forrest I, Parker CG. 2023. Proteome-wide fragment-based ligand and target discovery. *Isr. J. Chem.* 63:e202200098
37. Molina DM, Jafari R, Ignatushchenko M, Seki T, Larsson EA, et al. 2013. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science* 341:84–87
38. Savitski MM, Reinhard FB, Franken H, Werner T, Savitski MF, et al. 2014. Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science* 346:1255784
39. Peuget S, Zhu J, Sanz G, Singh M, Gaetani M, et al. 2020. Thermal proteome profiling identifies oxidative-dependent inhibition of the transcription of major oncogenes as a new therapeutic mechanism for select anticancer compounds. *Cancer Res.* 80:1538–50
40. Hwang HY, Kim TY, Szász MA, Dome B, Malm J, et al. 2020. Profiling the protein targets of unmodified bio-active molecules with drug affinity responsive target stability and liquid chromatography/tandem mass spectrometry. *Proteomics* 20:e1900325
41. Huber KV, Olek KM, Müller AC, Tan CSH, Bennett KL, et al. 2015. Proteome-wide drug and metabolite interaction mapping by thermal-stability profiling. *Nat. Methods* 12:1055–57
42. Dziekan JM, Yu H, Chen D, Dai L, Wirjanata G, et al. 2019. Identifying purine nucleoside phosphorylase as the target of quinine using cellular thermal shift assay. *Sci. Transl. Med.* 11:eaau3174
43. Perrin J, Werner T, Kurzawa N, Rutkowska A, Childs DD, et al. 2020. Identifying drug targets in tissues and whole blood with thermal-shift profiling. *Nat. Biotechnol.* 38:303–8
44. Tolvanen TA. 2022. Current advances in CETSA. *Front. Mol. Biosci.* 9:866764
45. Brummelkamp TR, Fabius AW, Mullenders J, Madiredjo M, Velds A, et al. 2006. An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors. *Nat. Chem. Biol.* 2:202–6
46. Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, et al. 2006. A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 441:106–10
47. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, et al. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343:84–87
48. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, et al. 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517:583–88
49. Biering SB, Sarnik SA, Wang E, Zengel JR, Leist SR, et al. 2022. Genome-wide bidirectional CRISPR screens identify mucins as host factors modulating SARS-CoV-2 infection. *Nat. Genet.* 54:1078–89
50. Deleted in proof

51. Neggers JE, Kwanten B, Dierckx T, Noguchi H, Voet A, et al. 2018. Target identification of small molecules using large-scale CRISPR-Cas mutagenesis scanning of essential genes. *Nat. Commun.* 9:502
52. Szlachta K, Kuscu C, Tufan T, Adair SJ, Shang S, et al. 2018. CRISPR knockout screening identifies combinatorial drug targets in pancreatic cancer and models cellular drug response. *Nat. Commun.* 9:4275
53. Deng X, Rong J, Wang L, Vasdev N, Zhang L, et al. 2019. Chemistry for positron emission tomography: recent advances in ^{11}C-, ^{18}F-, ^{13}N-, and ^{15}O-labeling reactions. *Angew. Chem. Int. Ed. Engl.* 58:2580–605
54. Goud NS, Bhattacharya A, Joshi RK, Nagaraj C, Bharath RD, Kumar P. 2021. Carbon-11: radiochemistry and target-based PET molecular imaging applications in oncology, cardiology, and neurology. *J. Med. Chem.* 64:1223–59
55. Korat S, Bidesi NSR, Bonanno F, Di Nanni A, Hoang ANN, et al. 2021. Alpha-synuclein PET tracer development—an overview about current efforts. *Pharmaceuticals* 14:847
56. Tang YZ, Booth TC, Bhogal P, Malhotra A, Wilhelm T. 2011. Imaging of primary central nervous system lymphoma. *Clin. Radiol.* 66:768–77
57. Barthel H, Seibyl J, Lammertsma AA, Villemagne VL, Sabri O. 2020. Exploiting the full potential of β-amyloid and tau PET imaging for drug efficacy testing. *J. Nucl. Med.* 61:1105–6
58. Wahl RL, Herman JM, Ford E. 2011. The promise and pitfalls of positron emission tomography and single-photon emission computed tomography molecular imaging-guided radiation therapy. *Semin. Radiat. Oncol.* 21:88–100
59. Pancholi K. 2012. A review of imaging methods for measuring drug release at nanometre scale: a case for drug delivery systems. *Expert Opin. Drug. Deliv.* 9:203–18
60. Liu J, Malekzadeh M, Mirian N, Song T-A, Liu C, Dutta J. 2021. Artificial intelligence-based image enhancement in pet imaging: noise reduction and resolution enhancement. *PET Clin.* 16:553–76
61. Moncada R, Barkley D, Wagner F, Chiodin M, Devlin JC, et al. 2020. Integrating microarray-based spatial transcriptomics and single-cell RNA-seq reveals tissue architecture in pancreatic ductal adenocarcinomas. *Nat. Biotechnol.* 38:333–42
62. Galeano Niño JL, Wu H, LaCourse KD, Kempchinsky AG, Baryiames A, et al. 2022. Effect of the intratumoral microbiota on spatial and cellular heterogeneity in cancer. *Nature* 611:810–17
63. Fan X, Dong J, Zhong S, Wei Y, Wu Q, et al. 2018. Spatial transcriptomic survey of human embryonic cerebral cortex by single-cell RNA-seq analysis. *Cell Res.* 28:730–45
64. Rodriques SG, Stickels RR, Goeva A, Martin CA, Murray E, et al. 2019. Slide-seq: a scalable technology for measuring genome-wide expression at high spatial resolution. *Science* 363:1463–67
65. Stickels RR, Murray E, Kumar P, Li J, Marshall JL, et al. 2021. Highly sensitive spatial transcriptomics at near-cellular resolution with Slide-seqV2. *Nat. Biotechnol.* 39:313–19
66. Ginsberg SD, Elarova I, Ruben M, Tan F, Counts SE, et al. 2004. Single-cell gene expression analysis: implications for neurodegenerative and neuropsychiatric disorders. *Neurochem. Res.* 29:1053–64
67. Raj A, van Oudenaarden A. 2008. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell* 135:216–26
68. Liu Y, Yang M, Deng Y, Su G, Enninful A, et al. 2020. High-spatial-resolution multi-omics sequencing via deterministic barcoding in tissue. *Cell* 183:1665–81.e18
69. Vickovic S, Lötstedt B, Klughammer J, Mages S, Segerstolpe Å, et al. 2022. SM-Omics is an automated platform for high-throughput spatial multi-omics. *Nat. Commun.* 13:795
70. Zou Y, Palte MJ, Deik AA, Li H, Eaton JK, et al. 2019. A GPX4-dependent cancer cell state underlies the clear-cell morphology and confers sensitivity to ferroptosis. *Nat. Commun.* 10:1617
71. Ennishi D, Jiang A, Boyle M, Collinge B, Grande BM, et al. 2019. Double-hit gene expression signature defines a distinct subgroup of germinal center B-cell-like diffuse large B-cell lymphoma. *J. Clin. Oncol.* 37:190–201
72. Moffitt JR, Lundberg E, Heyn H. 2022. The emerging landscape of spatial profiling technologies. *Nat. Rev. Genet.* 23:741–59
73. Larsson L, Frisen J, Lundeberg J. 2021. Spatially resolved transcriptomics adds a new dimension to genomics. *Nat. Methods* 18:15–18
74. Rao A, Barkley D, Franca GS, Yanai I. 2021. Exploring tissue architecture using spatial transcriptomics. *Nature* 596:211–20

75. Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, et al. 2009. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat. Methods* 6:377–82
76. Shapiro E, Biezuner T, Linnarsson S. 2013. Single-cell sequencing-based technologies will revolutionize whole-organism science. *Nat. Rev. Genet.* 14:618–30
77. Poulin J-F, Tasic B, Hjerling-Leffler J, Trimarchi JM, Awatramani R. 2016. Disentangling neural cell diversity using single-cell transcriptomics. *Nat. Neurosci.* 19:1131–41
78. Drokhlyansky E, Smillie CS, Van Wittenberghe N, Ericsson M, Griffin GK, et al. 2020. The human and mouse enteric nervous system at single-cell resolution. *Cell* 182:1606–22.e23
79. Enge M, Arda HE, Mignardi M, Beausang J, Bottino R, et al. 2017. Single-cell analysis of human pancreas reveals transcriptional signatures of aging and somatic mutation patterns. *Cell* 171:321–30.e14
80. Shin D, Lee W, Lee JH, Bang D. 2019. Multiplexed single-cell RNA-seq via transient barcoding for simultaneous expression profiling of various drug perturbations. *Sci. Adv.* 5:eaav2249
81. Habib N, Li Y, Heidenreich M, Swiech L, Avraham-Davidi I, et al. 2016. Div-Seq: single-nucleus RNA-Seq reveals dynamics of rare adult newborn neurons. *Science* 353:925–28
82. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. 2013. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat. Methods* 10:1213–18
83. Dixit A, Parnas O, Li B, Chen J, Fulco CP, et al. 2016. Perturb-Seq: dissecting molecular circuits with scalable single-cell RNA profiling of pooled genetic screens. *Cell* 167:1853–66.e17
84. Palla G, Fischer DS, Regev A, Theis FJ. 2022. Spatial components of molecular tissue biology. *Nat. Biotechnol.* 40:308–18
85. Tian L, Chen F, Macosko EZ. 2023. The expanding vistas of spatial transcriptomics. *Nat. Biotechnol.* 41:773–82
86. Mund A, Brunner A-D, Mann M. 2022. Unbiased spatial proteomics with single-cell resolution in tissues. *Mol. Cell* 82:2335–49
87. Geier B, Sogin EM, Michellod D, Janda M, Kompauer M, et al. 2020. Spatial metabolomics of in situ host–microbe interactions at the micrometre scale. *Nat. Microbiol.* 5:498–510
88. Ma S, Leng Y, Li X, Meng Y, Yin Z, Hang W. 2022. High spatial resolution mass spectrometry imaging for spatial metabolomics: advances, challenges, and future perspectives. *Trends Anal. Chem.* 159:116902
89. Djambazova KV, Klein DR, Migas LG, Neumann EK, Rivera ES, et al. 2020. Resolving the complexity of spatial lipidomics using MALDI TIMS imaging mass spectrometry. *Anal. Chem.* 92:13290–97
90. Rosenberg AB, Roco CM, Muscat RA, Kuchina A, Sample P, et al. 2018. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. *Science* 360:176–82
91. Marusyk A, Janiszewska M, Polyak K. 2020. Intratumor heterogeneity: the Rosetta stone of therapy resistance. *Cancer Cell* 37:471–84
92. González-Silva L, Quevedo L, Varela I. 2020. Tumor functional heterogeneity unraveled by scRNA-seq technologies. *Trends Cancer* 6:13–19
93. Zhang Y, Wang D, Peng M, Tang L, Ouyang J, et al. 2021. Single-cell RNA sequencing in cancer research. *J. Exp. Clin. Cancer Res.* 40:81
94. Patel AP, Tirosh I, Trombetta JJ, Shalek AK, Gillespie SM, et al. 2014. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 344:1396–401
95. Seferbekova Z, Lomakin A, Yates LR, Gerstung M. 2023. Spatial biology of cancer evolution. *Nat. Rev. Genet.* 24:295–313
96. Wang Y, Ma S, Ruzzo WL. 2020. Spatial modeling of prostate cancer metabolic gene expression reveals extensive heterogeneity and selective vulnerabilities. *Sci. Rep.* 10:3490
97. Ji AL, Rubin AJ, Thrane K, Jiang S, Reynolds DL, et al. 2020. Multimodal analysis of composition and spatial architecture in human squamous cell carcinoma. *Cell* 182:497–514.e22
98. Anderson AC, Yanai I, Yates LR, Wang L, Swarbrick A, et al. 2022. Spatial transcriptomics. *Cancer Cell* 40:895–900
99. McNamara KL, Caswell-Jin JL, Joshi R, Ma Z, Kotler E, et al. 2021. Spatial proteomic characterization of HER2-positive breast tumors through neoadjuvant therapy predicts response. *Nat. Cancer* 2:400–13
100. Chen B, Ma L, Paik H, Sirota M, Wei W, et al. 2017. Reversal of cancer gene expression correlates with drug efficacy and reveals therapeutic targets. *Nat. Commun.* 8:16022

101. Rotow J, Bivona TG. 2017. Understanding and targeting resistance mechanisms in NSCLC. *Nat. Rev. Cancer* 17:637–58
102. Sharma K, Schmitt S, Bergner CG, Tyanova S, Kannaiyan N, et al. 2015. Cell type– and brain region–resolved mouse brain proteome. *Nat. Neurosci.* 18:1819–31
103. Ortiz C, Carlén M, Meletis K. 2021. Spatial transcriptomics: molecular maps of the mammalian brain. *Annu. Rev. Neurosci.* 44:547–62
104. Beauchamp A, Yee Y, Darwin BC, Raznahan A, Mars RB, Lerch JP. 2022. Whole-brain comparison of rodent and human brains using spatial transcriptomics. *eLife* 11:e79418
105. Deng Y, Bartosovic M, Ma S, Zhang D, Kukanja P, et al. 2022. Spatial profiling of chromatin accessibility in mouse and human tissues. *Nature* 609:375–83
106. Chen WT, Lu A, Craessaerts K, Pavie B, Sala Frigerio C, et al. 2020. Spatial transcriptomics and in situ sequencing to study Alzheimer's disease. *Cell* 182:976–91.e19
107. Zeng H, Huang J, Zhou H, Meilandt WJ, Dejanovic B, et al. 2023. Integrative in situ mapping of single-cell transcriptional states and tissue histopathology in a mouse model of Alzheimer's disease. *Nat. Neurosci.* 26:430–46
108. Roth R, Kim S, Kim J, Rhee S. 2020. Single-cell and spatial transcriptomics approaches of cardiovascular development and disease. *BMB Rep.* 53:393–99
109. Martinez-Val A, Bekker-Jensen DB, Steigerwald S, Koenig C, Østergaard O, et al. 2021. Spatial-proteomics reveals phospho-signaling dynamics at subcellular resolution. *Nat. Commun.* 12:7113
110. Mao Y, Wang X, Huang P, Tian R. 2021. Spatial proteomics for understanding the tissue microenvironment. *Analyst* 146:3777–98
111. Sheng M, Greenberg ME. 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* 4:477–85
112. Salinas CBG, Lu TT-H, Gabery S, Marstal K, Alanentalo T, et al. 2018. Integrated brain atlas for unbiased mapping of nervous system effects following liraglutide treatment. *Sci. Rep.* 8:10310
113. Davoudian PA, Shao LX, Kwan AC. 2023. Shared and distinct brain regions targeted for immediate early gene expression by ketamine and psilocybin. *ACS Chem. Neurosci.* 14:468–80
114. Kim CK, Ye L, Jennings JH, Pichamoorthy N, Tang DD, et al. 2017. Molecular and circuit-dynamical identification of top-down neural mechanisms for restraint of reward seeking. *Cell* 170:1013–27.e14
115. Deguchi T, Iwanski MK, Schentarra E-M, Heidebrecht C, Schmidt L, et al. 2023. Direct observation of motor protein stepping in living cells using MINFLUX. *Science* 379:1010–15
116. Jungmann R, Avendaño MS, Dai M, Woehrstein JB, Agasti SS, et al. 2016. Quantitative super-resolution imaging with qPAINT. *Nat. Methods* 13:439–42
117. Wolff JO, Scheiderer L, Engelhardt T, Engelhardt J, Matthias J, Hell SW. 2023. MINFLUX dissects the unimpeded walking of kinesin-1. *Science* 379:1004–10
118. Scinto SL, Bilodeau DA, Hincapie R, Lee W, Nguyen SS, et al. 2021. Bioorthogonal chemistry. *Nat. Rev. Methods Primers* 1:30
119. Prokop S, Abranyi-Balogh P, Barti B, Vamosi M, Zoldi M, et al. 2021. PharmacoSTORM nanoscale pharmacology reveals cariprazine binding on Islands of Calleja granule cells. *Nat. Commun.* 12:6505
120. Bird RE, Lemmel SA, Yu X, Zhou QA. 2021. Bioorthogonal chemistry and its applications. *Bioconjug. Chem.* 32:2457–79
121. Tyler DS, Vappiani J, Cañeque T, Lam EY, Ward A, et al. 2017. Click chemistry enables preclinical evaluation of targeted epigenetic therapies. *Science* 356:1397–401
122. Sun DE, Fan X, Shi Y, Zhang H, Huang Z, et al. 2021. Click-ExM enables expansion microscopy for all biomolecules. *Nat. Methods* 18:107–13
123. Edgington LE, Verdoes M, Bogyo M. 2011. Functional imaging of proteases: recent advances in the design and application of substrate-based and activity-based probes. *Curr. Opin. Chem. Biol.* 15:798–805
124. Blum G, von Degenfeld G, Merchant MJ, Blau HM, Bogyo M. 2007. Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. *Nat. Chem. Biol.* 3:668–77
125. Hu M, Li L, Wu H, Su Y, Yang PY, et al. 2011. Multicolor, one- and two-photon imaging of enzymatic activities in live cells with fluorescently quenched activity-based probes (qABPs). *J. Am. Chem. Soc.* 133:12009–20

126. Yim JJ, Tholen M, Klaassen A, Sorger J, Bogyo M. 2017. Optimization of a protease activated probe for optical surgical navigation. *Mol. Pharmaceutics* 15:750–58
127. Deleted in proof
128. Verdoes M, Oresic Bender K, Segal E, van der Linden WA, Syed S, et al. 2013. Improved quenched fluorescent probe for imaging of cysteine cathepsin activity. *J. Am. Chem. Soc.* 135:14726–30
129. Kato D, Boatright KM, Berger AB, Nazif T, Blum G, et al. 2005. Activity-based probes that target diverse cysteine protease families. *Nat. Chem. Biol.* 1:33–38
130. Feng Y, Zhu S, Antaris AL, Chen H, Xiao Y, et al. 2017. Live imaging of follicle stimulating hormone receptors in gonads and bones using near infrared II fluorophore. *Chem. Sci.* 8:3703–11
131. Deng H, Lei Q, Yang N, Dai S, Peng H, et al. 2022. Expanded application of a photoaffinity probe to study epidermal growth factor receptor tyrosine kinase with functional activity. *Anal. Chem.* 94:10118–26
132. Kim E, Yang KS, Kohler RH, Dubach JM, Mikula H, Weissleder R. 2015. Optimized near-IR fluorescent agents for in vivo imaging of Btk expression. *Bioconjug. Chem.* 26:1513–18
133. Ofori LO, Withana NP, Prestwood TR, Verdoes M, Brady JJ, et al. 2015. Design of protease activated optical contrast agents that exploit a latent lysosomotropic effect for use in fluorescence-guided surgery. *ACS Chem. Biol.* 10:1977–88
134. Kelderhouse LE, Chelvam V, Wayua C, Mahalingam S, Poh S, et al. 2013. Development of tumor-targeted near infrared probes for fluorescence guided surgery. *Bioconjug. Chem.* 24:1075–80
135. Pang Z, Schafroth MA, Ogasawara D, Wang Y, Nudell V, et al. 2022. In situ identification of cellular drug targets in mammalian tissue. *Cell* 185:1793–805.e17
136. Choi SW, Guan W, Chung K. 2021. Basic principles of hydrogel-based tissue transformation technologies and their applications. *Cell* 184:4115–36
137. Ueda HR, Erturk A, Chung K, Gradinaru V, Chedotal A, et al. 2020. Tissue clearing and its applications in neuroscience. *Nat. Rev. Neurosci.* 21:61–79
138. Wang X, Allen WE, Wright MA, Sylwestrak EL, Samusik N, et al. 2018. Three-dimensional intact-tissue sequencing of single-cell transcriptional states. *Science* 361:eaat5691
139. Singh J, Petter RC, Baillie TA, Whitty A. 2011. The resurgence of covalent drugs. *Nat. Rev. Drug. Discov.* 10:307–17
140. De Vita E. 2020. 10 Years into the resurgence of covalent drugs. *Future Med. Chem.* 13:193–210
141. Boike L, Henning NJ, Nomura DK. 2022. Advances in covalent drug discovery. *Nat. Rev. Drug. Discov.* 21:881–98
142. Lawitz E, Mangia A, Wyles D, Rodriguez-Torres M, Hassanein T, et al. 2013. Sofosbuvir for previously untreated chronic hepatitis C infection. *N. Engl. J. Med.* 368:1878–87
143. Owen DR, Allerton CMN, Anderson AS, Aschenbrenner L, Avery M, et al. 2021. An oral SARS-CoV-2 M^{pro} inhibitor clinical candidate for the treatment of COVID-19. *Science* 374:1586–93
144. Nonaka H, Mino T, Sakamoto S, Oh JH, Watanabe Y, et al. 2023. Revisiting PFA-mediated tissue fixation chemistry: *FixEL* enables trapping of small molecules in the brain to visualize their distribution changes. *Chem* 9:523–40
145. Pan S, Jang SY, Wang D, Liew SS, Li Z, et al. 2017. A suite of "minimalist" photo-crosslinkers for live-cell imaging and chemical proteomics: case study with BRD4 inhibitors. *Angew. Chem. Int. Ed. Engl.* 56:11816–21
146. Nudell V, Wang Y, Pang Z, Lal NK, Huang M, et al. 2022. HYBRiD: hydrogel-reinforced DISCO for clearing mammalian bodies. *Nat. Methods* 19:479–85
147. Pan C, Cai R, Quacquarelli FP, Ghasemigharagoz A, Lourbopoulos A, et al. 2016. Shrinkage-mediated imaging of entire organs and organisms using uDISCO. *Nat. Methods* 13:859–67
148. Yang B, Treweek JB, Kulkarni RP, Deverman BE, Chen CK, et al. 2014. Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell* 158:945–58
149. Reich DS, Arnold DL, Vermersch P, Bar-Or A, Fox RJ, et al. 2021. Safety and efficacy of tolebrutinib, an oral brain-penetrant BTK inhibitor, in relapsing multiple sclerosis: a phase 2b, randomised, double-blind, placebo-controlled trial. *Lancet Neurol.* 20:729–38
150. Montalban X, Arnold DL, Weber MS, Staikov I, Piasecka-Stryczynska K, et al. 2019. Placebo-controlled trial of an oral BTK inhibitor in multiple sclerosis. *N. Engl. J. Med.* 380:2406–17

151. Healy LM, Stratton JA, Kuhlmann T, Antel J. 2022. The role of glial cells in multiple sclerosis disease progression. *Nat. Rev. Neurol.* 18:237–48
152. Lee DSW, Rojas OL, Gommerman JL. 2021. B cell depletion therapies in autoimmune disease: advances and mechanistic insights. *Nat. Rev. Drug. Discov.* 20:179–99
153. Hanker AB, Brewer MR, Sheehan JH, Koch JP, Sliwoski GR, et al. 2017. An acquired *HER2*^{T798I} gatekeeper mutation induces resistance to neratinib in a patient with HER2 mutant–driven breast cancer. *Cancer Discov.* 7:575–85
154. Kerbrat A, Ferre JC, Fillatre P, Ronziere T, Vannier S, et al. 2016. Acute neurologic disorder from an inhibitor of fatty acid amide hydrolase. *N. Engl. J. Med.* 375:1717–25
155. Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, et al. 2013. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N. Engl. J. Med.* 369:32–42
156. Wiczer TE, Levine LB, Brumbaugh J, Coggins J, Zhao Q, et al. 2017. Cumulative incidence, risk factors, and management of atrial fibrillation in patients receiving ibrutinib. *Blood Adv.* 1:1739–48
157. Skelly DA, Squiers GT, McLellan MA, Bolisetty MT, Robson P, et al. 2018. Single-cell transcriptional profiling reveals cellular diversity and intercommunication in the mouse heart. *Cell Rep.* 22:600–10
158. Litviňuková M, Talavera-López C, Maatz H, Reichart D, Worth CL, et al. 2020. Cells of the adult human heart. *Nature* 588:466–72
159. Xiao L, Salem JE, Clauss S, Hanley A, Bapat A, et al. 2020. Ibrutinib-mediated atrial fibrillation attributable to inhibition of C-terminal Src kinase. *Circulation* 142:2443–55