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Advances in Morphological Profiling of Cellular Responses to Environmental Stressors Using Cell Painting

JONNE RIETDIJK



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Abstract

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Humans are constantly exposed to stressors from natural sources, such as viruses and bacteria, and anthropogenic sources, including chemicals from consumer products and environmental pollution. The vast range and complexity of these exposures make it challenging to elucidate their potential adverse effects on human health. Morphological profiling has emerged as a powerful, scalable, and cost-effective approach for systematically studying diverse perturbations and their cellular responses. Cell Painting, an image-based assay for morphological profiling, measures global cellular responses through multiplexed staining of eight cellular components, high-content imaging, and multiparametric analysis. The resulting profiles capture subtle phenotypic changes, offering valuable insights into bioactivity and underlying biological mechanisms.

Among the diverse stressors humans face, emerging pathogenic viruses represent a particularly pressing global concern. To advance antiviral discovery, we adapted the Cell Painting assay by incorporating a virus-specific antibody, enabling simultaneous quantification of infection levels and in-depth characterization of host-cell responses. This approach offers clear advantages over conventional single-endpoint antiviral screens. Paper I investigated the effects of CoV-229E coronavirus infection on cell morphology and demonstrated the approach's utility for antiviral compound screening. Paper II expanded this with a 5,275-compound drug repurposing screen, identifying drugs that reverse SARS-CoV-2-induced cell phenotypes while providing insights into host-targeted pathways and compound mechanisms of action.

While viral pathogens pose significant public health challenges, environmental pollution represents a more chronic threat, contributing to an estimated nine million deaths annually. Despite this burden, chemicals are often inadequately assessed for health effects and typically studied individually, overlooking potential combination effects from co-exposure. To address this gap, Paper III applied Cell Painting to study the morphological effects of chemical mixtures. Using three environmental compounds, we demonstrated that Cell Painting effectively characterizes dose- and combination-dependent cellular responses across biologically diverse cell lines. In Paper IV, we profiled 900 environmental chemicals across multiple human cell lines to assess their bioactivity and infer putative mechanisms of action by comparing them to reference chemicals with well-characterized toxicity mechanisms.

Together, these findings establish Cell Painting as a powerful tool for capturing complex cellular responses across diverse contexts, offering new avenues for antiviral discovery and environmental hazard assessment.

Keywords: Cell Painting, Morphological profiling, safety assessment, antiviral screening

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The cell never acts; it reacts
- Ernst Haeckel.

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Rietdijk, J.**, Tampere, M., Pettke, A., Georgieva, P., Lapins, M., Warpman Berglund, U., Spjuth, O., Puumalainen, M. R., Carreras-Puigvert, J. (2021) A phenomics approach for antiviral drug discovery. *BMC Biology*, 19.
- II Asp, E.* , **Rietdijk, J.***, Tampere, M., Axelsson, H., Njenda, D., Potdar, S., Kalman, A., Georgieva, P., Lapins, M., Ballante, F., Soler, A., de Kort, M., Aittokallio, T., Zaliani, A., Kuzikov, M., Gribbon, P., Lo, D., Carreras-Puigvert, J., Seashore-Ludlow, B., Spjuth, O., Ostling, P. (Manuscript under review) A systematic host-centric approach to identifying repurposed drugs for viral infections. (*shared first authorship) Manuscript submitted
- III **Rietdijk, J*.**, Aggarwal, T*., Georgieva, P., Lapins, M., Carreras-Puigvert, J., Spjuth, O. (2022) Morphological profiling of environmental chemicals enables efficient and untargeted exploration of combination effects. *Science of the Total Environment*, 832:155058. (*shared first authorship)
- IV **Rietdijk, J.**, Hernandez-Valenzuela, S., Georgieva, P., Lapins, M., Carreras-Puigvert, J., Spjuth, O. Assessment of environmental chemicals through cell-based morphological profiling. Manuscript

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Additional Papers Not Included in the Thesis

In addition to the papers included in this thesis, the author has also contributed to the following publications:

- S-I Forsgren, E., **Rietdijk, J.**, Holmberg, D., Johansson, M., Carreras-Puigvert, J., Trygg, J., Lovell, G., Spjuth, O., Jonsson, P. (2025) Label-Free Live-Cell Imaging improves mode of action classification. *bioRxiv*, 2025.04.22.649936.
- S-II Frey, B., Holmberg, D., Bystrom, P., Bergman, E., Georgiev, P., Johansson, M., Hennig, P., **Rietdijk, J.**, Rosen, D., Carreras-Puigvert, J., Spjuth, O. (2025) Single-cell morphological profiling reveals insights into cell death. *bioRxiv*, 2025.01.15.633042.
- S-III Harrison, P. J., Gupta, A., **Rietdijk, J.**, Wieslander, H., Carreras-Puigvert, J., Georgiev, P., Wählby, C., Spjuth, O., Sintorn, I. M. (2023) Evaluating the utility of brightfield image data for mechanism of action prediction. *PLOS Computational Biology*, 19(7):e1011323.
- S-IV Braeuning, A., Balaguer, P., Bourguet, W., CarrerasPuigvert, J., Feiertag, K., Kamstra, J. H., Knapen, D., Lichtenstein, D., MarxStoelting, P., **Rietdijk, J.**, Schubert, K., Spjuth, O., Stinckens, E., Thedieck, K., van den Boom, R., Vergauwen, L., Von Bergen, M., Wewer, N., Zalko, D. (2023) Development of new approach methods for the identification and characterization of endocrine metabolic disruptors a PARC project. *Frontiers in Toxicology*, 5:1212509.

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Abbreviations

AB	Antibody
AOP	Adverse Outcome Pathway
CPE	Cytopathic Effect
COVID-19	Coronavirus Disease 2019
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DIPL	Drug-Induced Phospholipidosis
DMSO	Dimethyl Sulfoxide
EDC	Endocrine Disrupting Chemical
EPA	United States Environmental Protection Agency
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
HTS	High-Throughput Screening
ML	Machine Learning
MOA	Mechanism of Action
NAMs	New Approach Methodologies
NGRA	Next Generation Risk Assessment
OECD	Organisation for Economic Co-operation and Development
PCA	Principal Component Analysis
PLS-DA	Partial Least Squares Discriminant Analysis
POD	Point of Departure
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
UMAP	Uniform Manifold Approximation and Projection
WHO	World Health Organization
ECHA	European Chemicals Agency

1. Background

1.1 Historical Foundations of Morphology

The systematic study of the form and structure of plants and animals has been a central theme in biology since its earliest days, forming the basis for major scientific advances such as the taxonomic classification system developed by Carl Linnaeus in the eighteenth century and the theory of evolution introduced by Charles Darwin in the nineteenth century. The term *morphology* was first introduced by Goethe, who described it as "the science dedicated to the observation and description of everything" [1], referring broadly to the study of both the external form and internal structure of plants and animals. The invention of the microscope extended the scope of observation from whole organisms to the scale of tissues and cells. The development of staining methods, such as hematoxylin–eosin staining for histopathology, enabled the visualization of increasingly fine structural details, allowing for the detection of abnormalities and disease [2]. Since the early 2000s, innovations in high-throughput microscopy and computational analysis have reshaped microscopy into a data-rich discipline [3]. Instead of relying solely on human expertise, computers are now used to extract detailed descriptors, identify patterns that are often invisible to the human eye, and analyze image collections comprising thousands of samples.

1.2 High-Throughput Screening (HTS)

Biology is a complex web of interactions across molecules, cells, and tissues. To decipher this complexity, researchers systematically perturb biological systems by targeting individual components and observing the effects. Such perturbation-based studies help reveal the function of genes, pathways, and cellular processes. These approaches are particularly useful in drug discovery and toxicology, where understanding how chemical compounds affect biological systems is essential. In toxicology, the main goal is to detect chemicals with undesired or harmful effects, whereas in pharmacology, the focus is on discovering compounds with desired therapeutic activities.

While traditionally perturbations are assessed through low-throughput methods, modern drug discovery and toxicology require screening thousands to millions of perturbants simultaneously. To address this challenge, high-throughput screening (HTS) technologies utilizing robotics and automation have been developed. These systems employ miniaturized assay formats,

such as 384- or 1536-well plates, to efficiently test large chemical or genetic perturbation libraries [4]. These developments represent a major shift from hypothesis-driven "trial and error" approaches towards more systematic and data-driven discovery [5].

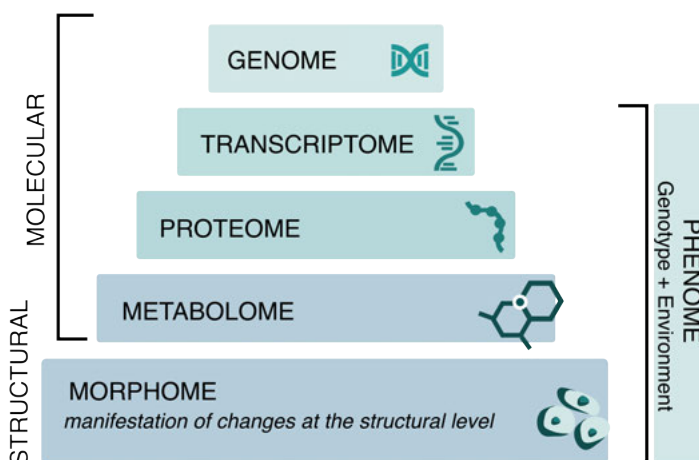


Figure 1.1. Overview illustrating the hierarchical relationship between the genome, transcriptome, proteome, and metabolome, and their connection to structural changes. The morphome reflects these molecular alterations at the structural level, together forming the phenome, which integrates both genetic and environmental influences.

1.3 Profiling Technologies

High-throughput screening (HTS) campaigns typically rely on well-established assay types that measure specific biochemical or cellular activities. The most common assays include: enzyme inhibition assays, reporter gene assays, cell viability assays, and measurements of key biomarkers such as ATP levels or caspase activation. While these approaches are powerful for screening large compound libraries against a specific target, they provide only a narrow view of the overall cellular response. To probe the full biological space, multiple targeted assays are often combined to form a battery of *in vitro* assays. These approaches quickly become costly and time-consuming, and they pose challenges for data integration [4].

To obtain a broader understanding of how perturbants influence cell state, researchers increasingly employ profiling technologies that capture high-dimensional and unbiased information from biological samples [6]. Profiling approaches differ from traditional screening approaches. Instead of

measuring a single readout, they measure hundreds to thousands of features from biological samples, enabling comprehensive characterization of biological systems without predefined hypotheses [6]. Among profiling modalities, transcriptomics and morphological cell profiling currently stand out for their scalability and affordability, enabling systematic profiling of thousands of perturbations [7].

1.4 Morphological Profiling

Image-based morphological profiling refers to the quantitative analysis of the morphology of cells and their organelles. It is a technique that aims at measuring hundreds to thousands of distinct properties from microscope images of cells to capture a wide range of cell states [8, 9]. Whereas molecular omics describe the composition of transcripts, proteins, or metabolites, morphology captures *how* these changes manifest at the structural level, from the subcellular level to tissue. To align with omics terminology, the term *morphomics* has been proposed for the systematic quantification of morphological descriptors across scales [10]. Moreover, the term *phenomics*, which has become increasingly used in the field of morphological profiling of cells, refers to the systematic, large-scale measurement of phenotypes [11] (Figure 1.1).

Image-based profiling offers a highly cost-effective alternative to molecular assays. It is estimated to be at least 1000 times cheaper than other single-cell omics such as transcriptomics and proteomics, and at least 15 times cheaper than bulk-omics methods [12]. Despite substantially lower costs, image-based approaches achieve predictive performance comparable to transcriptomics for MOA prediction tasks and provide complementary information, thereby enhancing the overall understanding of cellular states [7, 13].

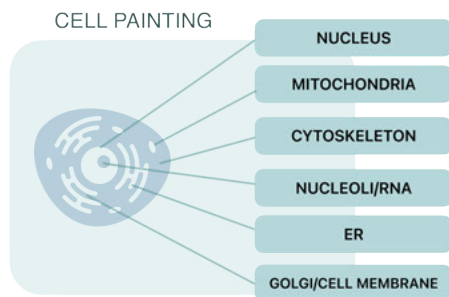


Figure 1.2. Overview of the eight cellular components targeted in the Cell Painting assay using six multiplexed fluorescent stains to visualize distinct subcellular structures and organelles, capturing comprehensive cellular phenotypes.

1.5 Cell Painting

Cell Painting is the most popular method for image-based morphological profiling. It is a multiplexed imaging assay that uses six fluorescent dyes to label eight distinct cellular components (Figure 1.2). The method was first introduced in 2013, and a detailed protocol was later published in 2016 by researchers at the Broad Institute of MIT and Harvard [8, 14].

In a typical Cell Painting experiment, biological samples are prepared in multi-well plates (e.g., 384-well plates) and exposed to perturbants of interest, for instance, chemical compounds, genetic perturbations, viruses, or physical stressors. After a defined incubation period, usually 24 or 48 hours, the cells are fixed, stained, and imaged using high-throughput microscopy. The resulting images are then processed using automated image analysis pipelines to extract quantitative features that are aggregated into morphological profiles (Figure 1.3). The resulting profiles can be analyzed with unsupervised methods to cluster and visualize phenotypic patterns, or with supervised models to classify perturbations by similarity, mechanism of action, or affected pathways [15]. Raw images can also be directly used in deep learning frameworks for predictive modeling [16]. The simplicity, versatility, and low cost of the assay have contributed significantly to its widespread adoption in both academia and pharmaceutical research for diverse purposes [15]. Below, I summarize the main applications of Cell Painting.

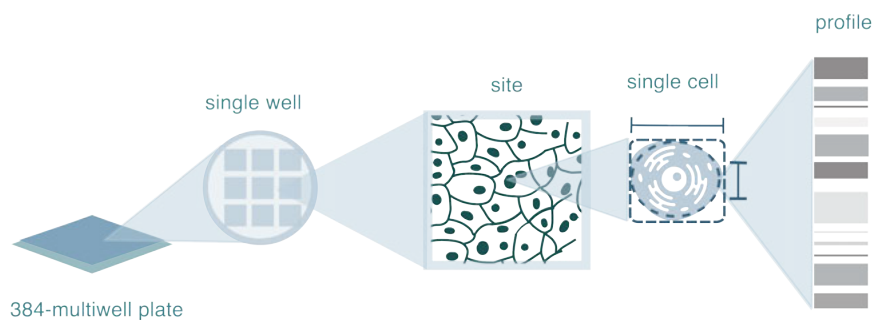


Figure 1.3. Overview of cell profiling at multiple scales. Images are captured from a 384-well plate, where each well contains multiple imaging sites. Individual cells within these sites are analyzed to extract morphological features, generating a unique quantitative profile that characterizes the cell’s phenotype.

1.6 Applications of Cell Painting

The most popular application of Cell Painting is in phenotypic drug discovery, where it is used to elucidate mechanisms of action (MOA) or detect off-target

effects. To do so, researchers typically screen large compound libraries to identify those that induce specific morphological changes. Compounds with similar morphological profiles are clustered together, allowing the identification of compounds with shared MOA independent of chemical structure or known biological targets [17].

Cell Painting is also a powerful method for identifying undesirable mechanisms of action and off-target effects. It can, for instance, reveal compounds that disrupt cholesterol homeostasis and lysosomal function [18, 19], cause mitochondrial dysfunction [20, 21, 22], autophagy [21], or induce cytoskeletal and microtubule disruption [23]. By incorporating reference compounds with known liabilities into screening campaigns, the assay can facilitate compound deprioritization by detecting unwanted effects, such as genotoxins, nonspecific electrophiles, and redox-active compounds that are unlikely to be viable as potential drugs [24].

Another valuable application involves identifying phenotypic signatures associated with disease, which can in turn be used to screen for compounds that restore cells to a healthy state. This strategy is particularly valuable for complex, multi-factorial diseases that lack a clear or easily measurable phenotype [14]. For example, the method has been used to detect patient-specific Parkinson's disease phenotypes in fibroblasts derived from affected individuals [25]. It has also enabled the characterization of allele-specific phenotypes in primary lung adenocarcinomas [26] and Alzheimer's disease [27], helping to uncover morphological signatures and to predict the functional effects of genetic variants in relation to clinical outcomes. This approach holds great promise for personalized medicine, as demonstrated by Hughes et al., who employed morphological profiling on genetically distinct cancer cell lines to identify drugs with patient-specific phenotypic responses [14, 28, 29].

In functional genomics, the assay has emerged as a powerful tool for studying gene function. By applying systematic gene perturbations, such as by RNA interference, CRISPR, or expression libraries, effects of specific genes and alleles can be studied [30, 31, 32, 33]. This strategy provides a scalable way to link genetic variants to cellular phenotypes and to identify small-molecule or biological modulators that target similar pathways. Large-scale efforts, such as the JUMP Cell Painting Consortium, have expanded this field by generating a morphology-based resource of over 20,000 genes, thereby uncovering gene-gene relationships and connecting perturbations to key biological processes [34, 35].

The method has also been demonstrated to be valuable for predicting the bioactivity of compounds with unknown or poorly characterized mechanisms of action. It has been utilized to annotate novel pseudo-organic molecules and evaluate the influence of stereochemistry and structural modifications on bioactivity [36, 37, 38, 39, 40, 41].

Beyond these applications, morphological profiling has become an increasingly popular approach for toxicity assessment and prediction. The method

has been applied to predict various toxicity endpoints, including mitochondrial toxicity [42, 20], cytotoxicity [43, 12], mutagenicity [44], and cell death mechanisms [45, 46]. It has also been used to predict rat acute oral toxicity [47] and organ-specific toxicity, such as hepatotoxicity [48, 49]. The approach has shown promise in safety and risk assessment of environmental chemicals, which will be discussed in greater detail in a later section. The main applications of Cell Painting are summarized in Figure 1.4.

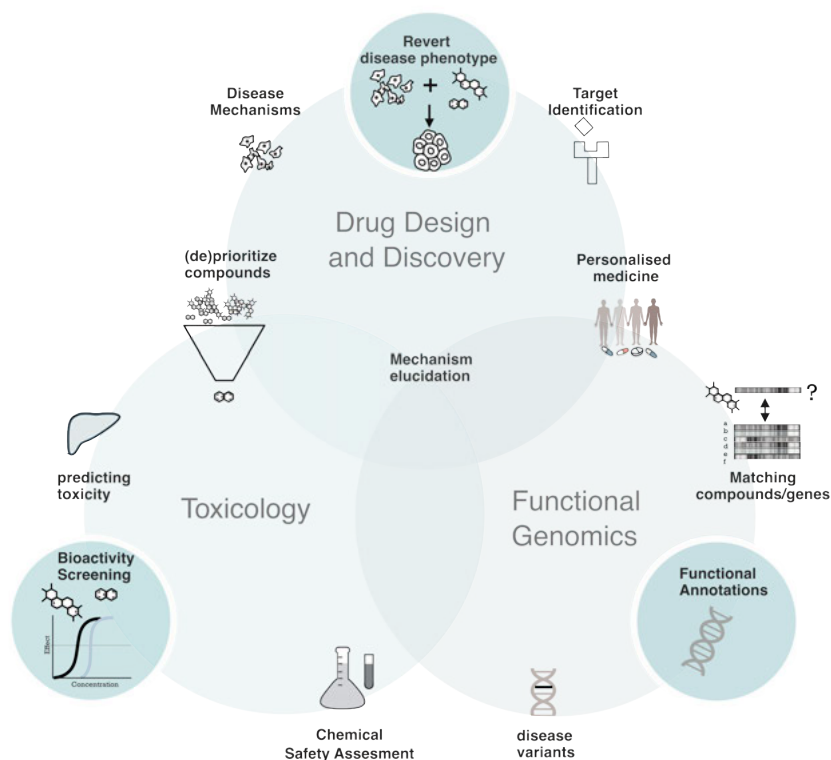


Figure 1.4. Cell Painting applications spanning drug discovery, toxicology, and functional genomics, including target identification, mechanism elucidation, and compound screening.

Methodological Innovations in Cell Painting

Since the start of this PhD project in early 2020, the Cell Painting assay has undergone a significant transformation, evolving into a versatile platform ca-

pable of capturing diverse cellular phenotypes across a wide range of biological systems. Initially, most Cell Painting studies closely followed the original protocol and were typically performed in relatively simple immortalized cell models such as U-2OS and A549 cells [8, 30, 50, 14, 51, 52, 53, 41, 37, 54, 36], HEK293T cells [31], MCF7 and other breast cancer cell lines [55, 56], and HeLa cells [40]. In recent years, however, there has been a clear shift toward more physiologically relevant models. Cell Painting is now increasingly applied to neuronal systems [57, 27], induced pluripotent stem cell (iPSC)-derived cells [58, 59], primary immune cell models such as human blood monocyte-derived macrophages and peripheral blood mononuclear cells (PBMCs) [60, 61, 62], primary human hepatocytes [12], and patient-derived fibroblasts [63, 64, 65]. The platform has also been extended beyond human cells, including implementations in insect [66] and fish cell models [67, 68], thereby broadening its applicability across species.

Alongside the diversification of cell models, several methodological adaptations of the original assay have emerged to address specific research aims. For instance, iterative staining protocols have enabled the inclusion of additional stains [69] and integration with optical pooled sequencing of molecular barcodes [35]. The LipocyteProfiler assay was developed using an optimized stain set to study metabolic diseases [70]. Additionally, Cell Painting has been combined with antibody-based detection to investigate viral infections, as presented in this thesis (Papers I and II)

From an analytical perspective, recent advances in Cell Painting data processing have greatly improved the extraction of biologically meaningful information from high-content images. Key developments include enhanced batch correction methods that improve comparability across experiments and platforms [71], deep learning-based segmentation tools such as CellPose [72], and self-supervised learning frameworks like DINO and DeepProfiler [73], which enable the direct extraction of high-dimensional features without requiring segmentation or manual feature engineering. Single-cell-level analyses have been further explored to capture cellular heterogeneity and reveal subpopulation-specific responses that are often masked in population averages [74, 75, 46]. There has also been active development of computational approaches to better distinguish true biological signals from noise and to facilitate the extraction of mechanistic insights from high-dimensional morphological data. These include frameworks for evaluating representation quality and biological relevance, such as mean Average Precision (mAP), subprofile analysis, and bioactivity hit-calling methods [76, 77, 78].

Finally, morphological profiling is expanding into temporal and spatial dimensions. Advances in time-resolved imaging and live-cell assays capture dynamic cellular responses over time and provide insights into transient or adaptive processes [79, 80, 81, 82], while three-dimensional imaging approaches have the potential to capture morphological signatures in more physiologically relevant 3D contexts [83].

2. Environmental Stressors

The majority of human diseases causing global morbidity, including cancers and metabolic disorders, cannot be explained by genetic factors alone. It is now widely recognized that the etiology of many chronic illnesses arises from complex gene-environment interactions [84]. These stressors encompass both natural sources, such as pathogenic viruses and bacteria, and anthropogenic sources, including pharmaceuticals and industrial chemicals. This thesis focuses on two sources of external stress in particular: pathogenic viruses and environmental chemical exposure.

2.1 Pathogenic Microorganisms

Infectious diseases have emerged unpredictably throughout human history [85]. The Black Death of the 14th century, for instance, killed an estimated one-third to one-half of Europe's population [86]. While advances in medicine and scientific understanding have substantially reduced the global burden of infectious disease, pathogens continue to pose major public health challenges [87]. Antibiotic-resistant bacteria represent a growing threat, acquiring resistance through genetic mutations and horizontal transfer of resistance plasmids. Bacterial antimicrobial resistance was associated with approximately 4.95 million deaths worldwide in 2019 [88]. Viral pathogens present equally significant challenges, causing hundreds of thousands of deaths annually [89]. The spread of viral pathogens is further intensified by population density, global travel, inadequate sanitation, and climate change, all of which contribute to an increased frequency of viral outbreaks [90, 91].

Antiviral Drug Discovery

Viral infections can be controlled through prevention (vaccines, prophylactic antivirals) or post-infection treatment (therapeutic antivirals) [92]. Small-molecule drugs and monoclonal antibodies have dramatically reduced morbidity and mortality for several major viral diseases, including hepatitis B and C, HIV, and herpes simplex virus [92]. However, over 200 human viral pathogens still lack approved therapeutic interventions [93]. RNA viruses are especially challenging targets due to their error-prone genomes, leading to rapid development of drug-resistant variants and increasing the risks of enhanced transmission and zoonotic spillover [94, 95].



Figure 2.1. Direct-acting antivirals (left) inhibit viral components directly, preventing viral replication but often facing rapid resistance development. In contrast, host-targeting antivirals (right) act on essential host-cell pathways required for viral propagation, reducing the likelihood of resistance but potentially increasing the risk of off-target effects.

Antiviral drugs can be classified into two main categories: direct-acting antivirals, which interact with viral targets, and host-directed antivirals, which target host-cell mechanisms (Figure 2.1). The majority of FDA-approved antiviral drugs function by directly targeting viral components. While these drugs often demonstrate high specificity for viral enzymes, they frequently lose efficacy due to the emergence of drug-resistant viral variants [96]. In contrast, host-directed antivirals exploit the specialized host-cell metabolic machinery that viruses use for propagation [97]. By targeting essential cellular pathways rather than individual viral enzymes, these antivirals offer a higher genetic barrier to resistance and often exhibit broad-spectrum activity against diverse viral families. However, this therapeutic approach carries an increased risk of off-target effects and cellular toxicity [98].

High-Throughput Screening for Antivirals

Cell-based assays are widely used for high-throughput screening of antiviral agents, for instance, to identify compounds that inhibit viral entry, genome replication, or assembly, or that prevent virus-induced cell death (Cytopathic Effect Inhibition (CPE) Assay) [99]. Despite their widespread adoption, current *in vitro* antiviral assays face multiple limitations. The CPE inhibition assay only provides an indirect readout of viral infectivity, which can lead to premature rejection of host-directed compounds that may themselves affect cell health [100]. Many methods require virus-specific antibodies or engineered reporter cell lines, limiting their use during emerging viral outbreaks. Finally, conventional approaches offer little mechanistic insight into virus-host interactions, slowing the identification of therapeutic targets and rational antiviral design.

2.2 Chemical Stressors

Chemicals in the Environment

The Industrial Revolution has led to a dramatic increase in the number of chemicals released and accumulated in the environment, including industrial chemicals, food additives, pesticides, and pharmaceuticals [101]. The global production of chemicals continues to rise, reaching levels exceeding the world's capacity for adequate assessment and monitoring [102]. Comprehensive toxicological information exists for only a small fraction of the more than 140,000 chemicals currently in commerce [103, 104], as illustrated in Figure 2.2. The European Environment Agency (EEA) estimated that only 0.5% of chemicals on the market are extensively characterized, whereas around 10% are fairly well characterized, 20% have limited hazard and exposure information, and approximately 70% remain poorly characterized for their hazard and exposure [105]. This underscores the urgent need for methods capable of rapidly screening the expanding universe of chemicals to protect both human and environmental health.

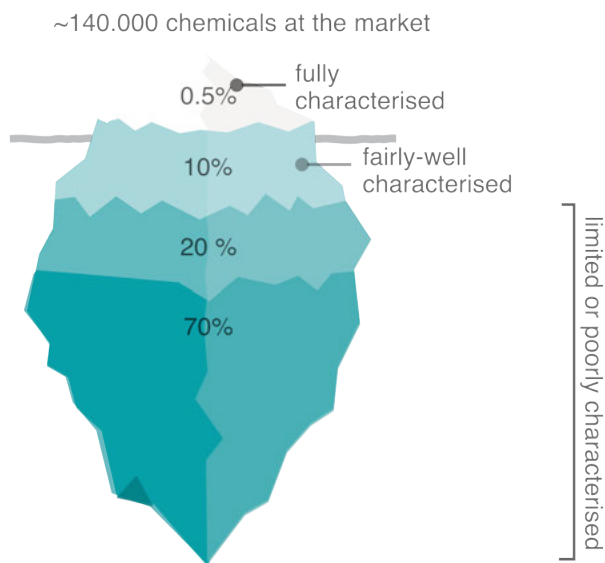


Figure 2.2. The iceberg illustrates how only a small fraction of chemicals are well characterized for hazard and exposure, while the majority remain insufficiently studied. Data from the European Environment Agency (2019) [106].

Chemical Exposures and Health

Chemical pollution poses serious risks to ecosystems and human health worldwide. Human-made environmental contaminants are estimated to contribute to 16% of premature deaths globally, with pollution-related diseases accounting for more than one in six deaths [107, 108]. Chemical exposure has been associated with a wide range of developmental disorders and diseases, including cancers [109], metabolic disorders [110], cardiovascular diseases [111], and reduced fertility [112]. Environmental exposures play a critical role in the etiology of common chronic diseases, which constitute a major and growing global health burden [113].

Combination Effects

Real-world exposures to environmental chemicals involves complex mixtures of hundreds to thousands of chemicals that vary across time and space. Traditional risk assessment, however, has predominantly focused on evaluating single chemicals in isolation. Even when the toxic effects of individual chemicals are negligible, their combined effects can reach biologically relevant or toxicologically relevant levels, a phenomenon known as the "Something from Nothing" effect [114]. Current mixture risk assessments, including those adopted by regulatory agencies, typically rely on toxicity data for individual components to predict overall mixture toxicity.

However, component-based approaches may substantially misestimate the risks of complex mixtures [115]. Combined effects are typically described by two main models: dose addition (DA), which assumes similar modes of action, and independent action (IA), which assumes dissimilar modes of action [116]. DA assumes that one compound can be substituted by an equivalent fraction of an equally potent dose of another without altering the overall effect. However, real-world exposures do not always conform to the dose addition paradigm. Interactions can occur between chemicals, either through direct molecular interactions or through a shared biological target. This can modify toxicity and produce effects that exceed those expected from the individual components, a phenomenon known as synergy or potentiation [115]. Synergistic interactions have been reported in several contexts, for example, between heavy metals and pesticides, which can amplify toxic effects, and among multiple weakly estrogenic chemicals that collectively result in significant endocrine activity [114].

Chemical Safety Assessment

Most chemicals on the market today are still evaluated using animal toxicity studies. These traditional approaches raise ethical concerns and are costly, time-consuming, limited in mechanistic insight, and often difficult to extrap-

olate across species [117]. To overcome these limitations, New Approach Methodologies (NAMs), which are defined as "technologies and methods that generate chemical hazard information without using intact animals", are being developed and implemented. Next Generation Risk Assessment (NGRA) provides a framework for integrating NAMs into chemical risk assessment to support more human-relevant, mechanistic-driven, and efficient evaluations of chemical safety [118]. NAMs have driven the development of high-throughput *in vitro* approaches for chemical safety assessment. The U.S. Environmental Protection Agency (EPA) ToxCast and the Tox21 programs exemplify this shift, using batteries of a large number of targeted assays to evaluate tens of thousands of chemicals. These large-scale initiatives generate valuable mechanistic data for predictive toxicology but remain limited by cost, variability, and challenges in data integration [4].

To expedite the assessment of the ever-growing number of chemicals under regulation, agencies such as the European Chemicals Agency (ECHA) are increasingly applying read-across approaches, which infer toxicity from structurally similar substances. While this strategy accelerates evaluation and reduces testing needs, it risks overlooking differences in biological activity arising from subtle structural variations [119]. These limitations have already led to so-called *regrettable substitutions*, such as the replacement of Bisphenol A with Bisphenol S, which, despite reduced estrogen receptor binding, exhibited unexpectedly higher affinity for other receptors, creating new endocrine-disrupting effects [119]. Therefore, integrating biological data from high-content or omics-based assays could greatly enhance the reliability of structure-based risk assessment.

Cell Painting in the Context of Safety Assessment

The EPA was among the first to recognize the potential of Cell Painting for chemical safety and risk assessment, integrating it into a two-tiered new approach methodology (NAM) framework [22]. This framework assesses Cell Painting-based high-throughput phenotypic profiling (HTPP) together with high-throughput transcriptomics as a first-tier assessment designed to (1) identify threshold concentrations that perturb biological processes and (2) elucidate putative mechanisms of action. Nyffeler and colleagues demonstrated that Cell Painting could be used to predict bioactivity of a large set of environmental chemicals, with toxicokinetic modeling revealing good concordance between *in vitro* estimates and established *in vivo* effect values [54, 22].

Since then, a growing number of studies have employed Cell Painting in the context of chemical safety assessment. For instance, it has been used to investigate the effects of nanoparticles [120] in liver cells, cobalt nanoparticles in macrophages [121], surface-coated silver nanoparticles [122], and PFAS mixtures [123, 124]. A recent study on p,p'-DDE, a persistent environmental

contaminant derived from the breakdown of the pesticide DDT, showed that it induced effects similar to those elicited by a Wnt-pathway activator [125]. Cell Painting applied to fish cells further demonstrates its potential to replace or reduce in vivo testing [67, 68]. The work in this thesis extends this research through two studies on environmental chemicals. Paper III investigates combination effects of co-exposure to environmental chemicals using Cell Painting. Paper IV presents a large-scale screen of environmental chemicals across multiple cell lines, comparing responses across cellular contexts and against well-annotated reference compounds to elucidate putative mechanisms of action. Collectively, these studies highlight Cell Painting's potential to characterize diverse chemical classes and toxicity mechanisms, and reduce reliance on in vivo testing for environmental chemical safety assessment.

3. Aims

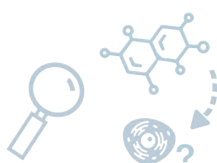
The overarching aim of this thesis was to evaluate how Cell Painting can be applied within the contexts of antiviral screening and chemical risk assessment.

To address this overarching goal, we set out the following specific aims:

1. **Establish Cell Painting for virology and antiviral screening** (*Papers I and II*). Investigate how morphological profiling can be used to reveal host-cell responses to viral infection and to identify antiviral compounds that reverse infection-induced phenotypes.
2. **Apply morphological profiling to chemical safety assessment** (*Paper IV*). Use morphological profiling to determine cellular bioactivity and to explore mechanisms of action of understudied environmental chemicals.
3. **Characterize combination effects of environmental chemicals using Cell Painting** (*Paper III*). Assess how morphological features capture the effects of combined chemical exposures across diverse cell models.



Establish Cell Painting for virology and antiviral screening



Apply morphological profiling to chemical safety assessment



Characterize chemical combination effects using Cell Painting

4. Methodologies

This chapter outlines the general methods used in this thesis. The work is built upon the Cell Painting assay, a multiplexed, high-content imaging technique that enables the quantification of thousands of morphological features from microscopy images of cells. For the work described here, we adapted the protocol described by Bray et al. and Cimini et al. [14, 126] to address the research questions of interest and tailored the workflow to the specific experimental setup and available instrumentation. Although specific parameters vary between individual studies and are detailed in the respective manuscripts, the general workflow is the same (Fig. 4.1). This chapter summarizes the main methodological steps and is organized into three stages: experimental design and automation, image acquisition and processing, and multivariate data analysis.

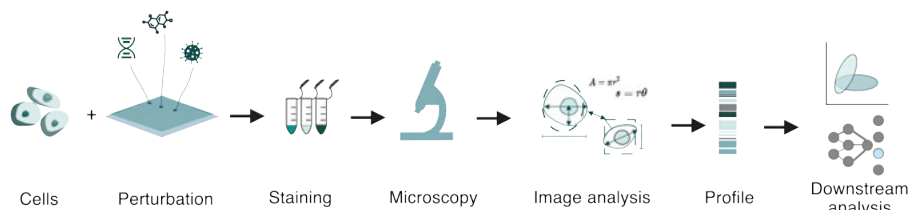


Figure 4.1. Overview of the standard steps in a morphological profiling pipeline. The workflow typically involves culturing cells, applying perturbations, staining cellular components, and capturing images using high-throughput microscopy. These images are then analyzed to extract quantitative morphological profiles, which are subsequently used for various downstream analyses.

4.1 Experimental Design and Automation

Selection of Cell Model

The first step in assay design involves selecting a suitable cell model relevant to the research question. The original Cell Painting assay was established using human osteosarcoma cells (U-2 OS). A key advantage of these cells is that they are well-characterized and robust for high-content imaging. For this thesis, the suitability of a cell model was assessed using the following criteria:

Table 4.1. Overview of main cell lines used in this thesis, their species, sex, origin, and cell type.

Cell line	Species	Sex	Origin	Cell type
U-2 OS	Human	Female	osteosarcoma	Epithelial
A549	Human	Male	adenocarcinoma	Epithelial
MCF7	Human	Female	breast cancer	Epithelial
Caco-2	Human	Male	Colorectal adenocarcinoma	Epithelial
HepG2	Human	Male	Hepatocellular carcinoma	Epithelial
VERO-E6	Monkey	Female	Kidney (continuous line)	Epithelial
A549-ACE2	Human	Male	adenocarcinoma (engineered)	Epithelial
MRC-5	Human	Male	Fetal lung	Fibroblast

1. Cells should form a flat, uniform monolayer without clumping when cultured in multi-well plates.
2. Single cells must be separable using image analysis tools, allowing reliable separation of foreground and background and robust single-cell segmentation.
3. Cells should display consistent morphological responses to a set of positive controls (i.e., those inducing pronounced morphological effects in cells).

For each cell line included in this thesis, cell density optimization was performed. In general, a confluency of 70–80% yields the most consistent morphological profile results. The main cell models used in this thesis are summarized in Table 4.1. Notably, recent advances in deep learning have greatly facilitated image segmentation, enabling the inclusion of more complex and heterogeneous cell types that were previously difficult to analyze.

Selection of Doses and Replicates

Perturbations used in this thesis included small-molecule drugs and environmental chemicals. Both were dissolved in DMSO. Treatment concentrations and durations were optimized for each study to capture phenotypic changes while minimizing toxicity, which can obscure compound-specific morphological effects. Treatment duration was experiment-specific, either 24 or 48 hours. Chemicals were pre-spotted onto assay plates using either an Open-trons liquid-handling robot, an I.DOT non-contact liquid dispenser, or an Echo acoustic dispenser. For the projects involving environmental chemicals, handling procedures were further optimized to improve solubility of environmental chemicals, including additional mixing steps and direct spotting of the compounds onto the cells to ensure uniform exposure.

Replicates are essential for ensuring robust and reproducible phenotypic profiling data. In this work, both technical and biological replicates are included to capture variation from different sources. Technical replicates (i.e.,

samples placed on the same assay plate) help reduce intra-plate variation, while biological replicates (i.e., samples placed on separate plates and preferably derived from independent cell cultures) are included to capture biological variability and enhance statistical robustness.

Placement of Samples and Controls

Image-based screens are sensitive to experimental biases, such as edge and positional effects [16]. To mitigate these, we used a constraint-programming-based plate layout tool [127] that effectively distributes treatments and controls across multi-well plates, thereby reducing positional confounding effects and improving analytical robustness. Unlike targeted bioassays with defined positive controls (e.g., those targeting the receptor of interest), profiling assays typically lack such controls due to their multi-parametric nature. Instead, it is common to include phenotypic reference compounds that induce distinct and reproducible morphological changes across cell types [8, 128]. In our experimental design, we included three to five control compounds to assess the assay performance and reproducibility. Solvent-treated samples (DMSO) were used as negative controls to establish baselines for normalization.

Multiplexed Staining

The assay uses a panel of six fluorescent dyes to label eight key cellular components, as summarized in the table below 4.2. The standard staining procedure involves four key steps: live-cell staining with MitoTracker, fixation, permeabilization, and post-fixation staining with the Cell Painting stains. Permeabilization and staining can be combined to simplify the assay [129].

Table 4.2. *Cellular compartments stained in the Cell Painting assay and their corresponding fluorescent stains*

Cellular Compartment / Organelle	Fluorescent Dye
Nucleus	Hoechst
Nucleoli and Cytoplasmic RNA	Syto
Actin Cytoskeleton	Phalloidin
Endoplasmic Reticulum	Concanavalin A
Golgi Apparatus and Plasma Membrane	Wheat Germ Agglutinin
Mitochondria	MitoTracker

Combining Immunohistochemistry with Cell Painting

For papers I and II, we developed an adaptation of the original protocol to include virus-specific antibodies. To accommodate this, the MitoTracker live-cell staining step was omitted, simplifying the workflow in biosafety level 3

(BSL-3) laboratories. The workflow comprised the following steps: (1) fixation and washing of infected and drug-treated cells, (2) blocking with fetal bovine serum (FBS) or bovine serum albumin (BSA), (3) permeabilization using Triton X-100, (4) overnight incubation with primary antibody (AB), (5) incubation with the secondary AB and Cell Painting dyes.

Automating the Cell Painting Assay

To improve reproducibility and throughput, several steps of the Cell Painting assay were progressively automated during the PhD project. Initially, liquid handling was carried out using automated multi-channel pipettes. In subsequent years, additional automation was introduced, including automated reagent dispensers (Biotek MultiFlo FX), microplate washers (Biotek 405 LS and BlueWasher), and advanced liquid handling systems (Opentrons OT-2, Flowbot One, I.DOT, and Echo). An automated setup, using a robotic arm (UR3), was developed within the Pharmb.io research group for automated plate transport between the plate hotel, washer, and dispensers. The robot is controlled through custom scheduling software, enabling a fully automated workflow from liquid handling to plate washing and imaging preparation.

4.2 Image Acquisition and Processing

High-throughput Microscopy

In this thesis, two automated microscope systems were used; the specifications are summarized in table 4.3. Fluorescent images were captured using five fluorescent channels. Exposure times were optimized for each channel to maximize the dynamic range without reaching saturation. For each well, between four and nine sites of view were captured, using a single z-plane. Images were saved as 12-bit gray-scale TIFF files without binning.

Table 4.3. *Microscope specifications and imaging parameters used for fluorescence acquisition. Numerical aperture (NA)*

	ImageXpress Micro XLS	Squid [130]
Vendor	Molecular Devices	Cephla
Depth of field	Widefield	Widefield
Objective	20X, NA 0.45	20X, NA 0.5
Focus	Laser-based	Laser-based
Bit depth	12-bit	12-bit
Fluorescent channels	5	5

Image Analysis Pipeline

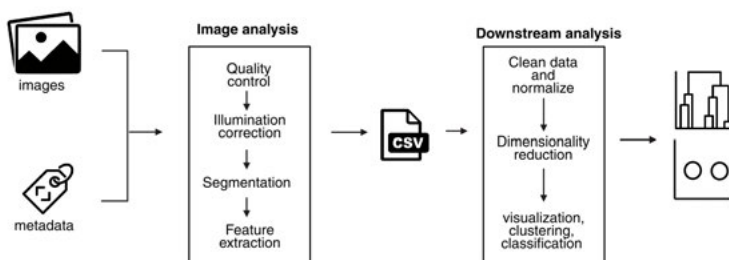


Figure 4.2. Images and accompanying metadata are processed through an image analysis pipeline, including quality control, illumination correction, segmentation, and feature extraction to generate quantitative feature data. The resulting dataset is cleaned, normalized, and analyzed through dimensionality reduction, clustering, and visualization to reveal phenotypic patterns.

The large volume of image data was processed using an automated pipeline built in CellProfiler [131], including an image quality pipeline, illumination correction, and feature extraction pipeline.

Quality control

An essential step in image-based assays is a quality control (QC) pipeline to detect images with artifacts which may corrupt the data with false values [16]. Given the large volume of data (usually thousands/millions of images), manual inspection is not possible. We set up an automated quality control pipeline to identify and exclude images with artifacts (e.g., out-of-focus images, debris, condensation). Image QC metrics were extracted using CellProfiler and included FocusScore, MaxIntensity, MeanIntensity, PercentMaximal, PowerLogLogSlope, and StdIntensity. Images deviating by more than five or ten standard deviations from the plate median, or meeting thresholds for saturation or blurriness, were excluded.

Illumination correction

To correct for uneven illumination in the images, a polynomial illumination correction function was calculated for each plate and image channel across all cycles, resulting in one illumination correction image per channel. These illumination functions were then applied in the analysis pipelines to correct the raw images by dividing each image by its corresponding illumination correction image.

Segmentation

The Hoechst staining was used for nuclei segmentation, while the cytoskeleton staining or cytoplasmic RNA was used for whole-cell segmentation, dependent on the cell line. In this thesis, both classical image analysis and deep learning-based methods were used for segmenting nuclei, cytoplasm, and whole cells. Classical methods employed Otsu thresholding for nuclei and watershed segmentation based on minimum cross-entropy of cytoplasmic RNA/actin staining, with thresholds adjusted per cell line and experiment. In later projects, CellPose was integrated into the pipeline, needing minimal fine-tuning across cell types.

Feature extraction

Following segmentation, the single cell masks were used to extract quantitative features describing size, shape, intensity, and texture across all 5 channels. Using CellProfiler, we extracted between 2,000 and 5,000 morphological features per cell and exported them in CSV or parquet format for downstream analysis. A distributed system was set up to parallelize image processing using high-performance computing infrastructure.

4.3 Multivariate data analysis

Feature extraction from Cell Painting images yields high-dimensional data matrices. A typical morphological profiling experiment testing 100 compounds can generate over a billion data points. Given this scale and complexity, multivariate data analysis is essential for identifying meaningful patterns and correlations. To transform high-dimensional data into interpretable morphological profiles, several preprocessing steps are applied to aggregate, normalize, and standardize the morphological features. The following general workflow was used:

Generation of Morphological Profiles

1. **Aggregation:** Single-cell measurements were aggregated at the well level by computing the median (or mean) value of each feature across all cells within a well.
2. **Quality Filtering:** Images flagged during quality control or containing very low cell counts were excluded from further analysis.
3. **Normalization:** Image-level profiles were normalized using median absolute deviation (MAD) normalization relative to the DMSO controls in the assay. This step ensured that features were comparable across plates and batches.

4. **Feature Selection:** Features exhibiting minimal variability ($SD < 0.001$), excessive variability ($SD > 10,000$), or missing values were excluded from further analysis.
5. **Replicate Aggregation:** Replicate wells corresponding to the same perturbation were averaged to generate a single perturbation-level profile.

Unsupervised Machine Learning methods

Hierarchical clustering

Unsupervised hierarchical clustering was applied to reveal underlying structure and relationships in the data. Clustering was performed using Euclidean distance and Ward's linkage, which minimizes within-cluster variance at each merging step to create compact, well-separated clusters. This approach enabled us to detect similarity between treated samples and control groups, and to compare phenotypic effects across different compounds

Principal Component Analysis (PCA)

Principal component analysis (PCA) is a statistical technique used to reduce the dimensionality of large datasets by transforming them into a smaller set of orthogonal components that retain most of the original variance. It does so by decomposing the data into latent variables that are linear combinations of the original features, where the corresponding loadings indicate how strongly each feature contributes to each principal component. In this thesis, PCA was applied for exploratory data analysis to detect batch effects, visualize high-dimensional data in lower-dimensional space, identify compound-induced phenotypic effects, and determine which features contributed most to variation across samples.

Uniform Manifold Approximation and Projection (UMAP)

UMAP was used to explore potential clusters in the data that might not be captured by linear methods such as PCA. Default parameters were used unless specified otherwise.

Supervised Machine Learning methods

Partial least-squares discriminant analysis (PLS-DA)

Partial Least-Squares Discriminant Analysis (PLS-DA) is a supervised multivariate method that projects high-dimensional data into a lower-dimensional space to maximize separation between predefined classes. Unlike Principal Component Analysis (PCA), which is unsupervised and maximizes variance within the explanatory variables (X), PLS-DA incorporates class labels (Y) to find latent variables that maximize the covariance between X and Y . PLS-DA

can be seen as a supervised version of PCA, achieving dimensionality reduction while simultaneously optimizing class discrimination [132]. In paper I, PLS-DA was used to train a model that classifies infection status based on cell morphology features and to identify feature classes associated with viral infection. In paper II, we computed mean prediction scores for a large number of drugs to identify compounds that could revert an infection-associated phenotype. In paper III, PLS-DA was employed as a measure of morphological activity by calculating the PLS-DA distance from DMSO, to compare the effects of single and combined exposure to chemicals.

Perturbation Strength and Quality

The high content of the data presents challenges in evaluating phenotypic activity and ensuring consistency across samples, as it is difficult to separate true biological signals from baseline noise. In this thesis, we used various methods to quantify perturbation strength and consistency. In paper II, we used the induction score as a measure of bioactivity, as described earlier [41]. The induction score quantifies the overall strength of a morphological perturbation and is defined as the percentage of features that change upon treatment compared to DMSO controls. We used a threshold of $|Z\text{-score}| > 1.96$ relative to the DMSO controls per plate. A limitation of this measure is that it does not account for how consistent the perturbations are and is therefore prone to positional bias and outliers. In papers II and IV, we used PLS-DA scores to measure the relative strength of perturbation to compare concentrations for each compound and to compare single and combined effects of chemicals. In paper IV, we use PLS-DA distance trained on infected vs non-infected to get a measure of how close the morphology is to either of the two populations. In papers III and IV, we used mean average precision (mAP) [76] to assess intra- and inter-group similarities. This metric was used both to identify significant perturbations and to evaluate the phenotypic consistency of groups sharing the same MOA.

Biological Interpretation of Features

Individual Cell Painting features can be challenging to interpret on their own. To obtain more biologically meaningful insights, features can be grouped into functionally related categories. Here, I established a grouping of features and visualization to capture broad biological changes better while remaining as concise as possible. The features were organized according to cellular component and feature group, such as intensity, radial distribution and granularity. Certain feature groups with limited biological interpretability were combined into broader categories, such as correlation features, whereas easier interpretable groups, such as those describing cell area, nuclear count, and

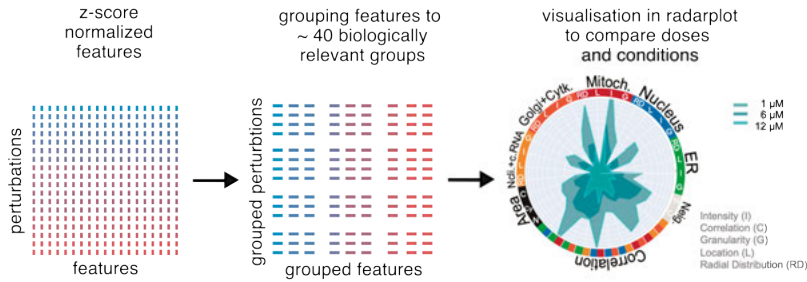


Figure 4.3. Radar plot visualization of grouped morphological features. Z-score normalized features are aggregated into biologically meaningful categories, enabling more intuitive comparison of cellular responses across doses and conditions.

multinucleation, are shown separately due to their clear interpretability and relevance for assessing cytotoxicity or proliferative responses. Correlation features were grouped by imaging channel, as the direction of the correlation is not meaningful in this context. This organization and its accompanying visualization facilitate the exploration of distinct morphological patterns and dose-dependent effects, enabling intuitive comparisons across cell lines, compounds, and exposure levels (Fig 4.3).

5. Summary of Papers

5.1 Paper I: A phenomics approach for antiviral drug discovery

The COVID-19 pandemic underscored the urgent need for innovative strategies to accelerate antiviral drug discovery against emerging pathogenic viruses. Conventional screening approaches primarily target viral proteins or assess cell viability, often overlooking the complex host-cell responses to infection and treatment. To address this limitation, in paper I we developed a novel phenomics approach that integrates morphological profiling with antibody-based detection of viral infection in a single image-based assay (Fig. 5.1). Specifically, we adapted the Cell Painting protocol by substituting the mitochondrial dye with an antibody against the coronavirus nucleoprotein, allowing simultaneous detection of infected cells while capturing in-depth morphological profiles reflecting the host-cell state.

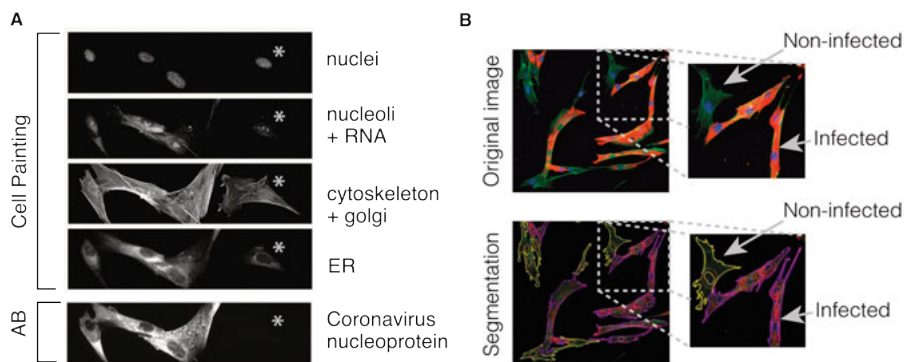


Figure 5.1. (A) Image examples of MRC-5 human lung fibroblasts infected with coronavirus 229E stained with Cell Painting stains along with a coronavirus nucleoprotein antibody. (B) A custom-built CellProfiler image analysis pipeline segments individual cells and classifies them as infected or non-infected.

Cell Painting captures a virus-induced phenotype

The first question we addressed was whether Cell Painting could detect a virus-induced phenotype. To investigate this, we infected MRC-5 primary lung fibroblasts with human coronavirus 229E (CoV-229E). Viral infection induced

pronounced morphological changes, which were captured by Cell Painting. In particular, we observed significant alterations in the ER and cytoplasmic RNA/nucleoli staining, reflecting disruptions of known host-cellular machinery hijacked by Coronaviruses.

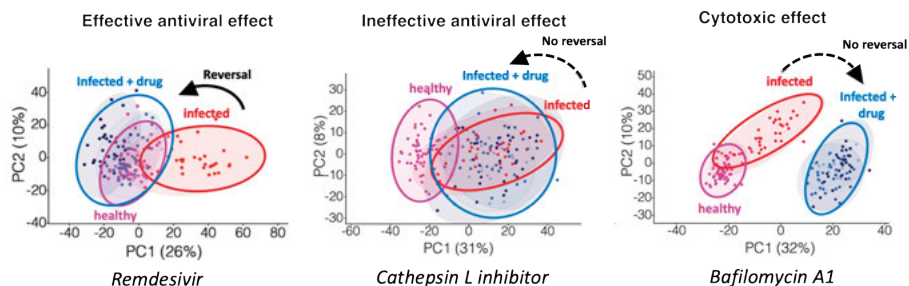


Figure 5.2. Principal component analysis illustrates three outcomes: (i) an effective antiviral, where the infected cell phenotype is reversed toward healthy morphology; (ii) an ineffective antiviral, where the infected phenotype is not reversed; and (iii) a cytotoxic compound where the drug-treated cells deviate from both healthy and infected phenotypes.

Identification of antiviral compounds that reverse the phenotype

Next, we demonstrated how this platform can be applied to screen for antiviral agents by identifying compounds that reverse the virus-induced phenotype. A panel of nine compounds was screened, including six established antivirals and three novel host-targeting candidates (TH3289, TH6744, TH5487). Principal component analysis showed that effective antivirals, particularly Remdesivir and E-64d, reversed the morphological profiles toward non-infected states, whereas non-effective antivirals did not reverse the phenotype and toxic compounds deviated from both (Fig. 5.2). Notably, the three novel candidates showed dose-dependent antiviral activity but also reduced cell viability. Such compounds would likely be discarded in conventional CPE assays, highlighting an advantage of our approach for identifying host-targeting antivirals.

Mechanistic insights into compounds

Beyond quantifying similarity to non-infected and infected states, morphological profiles also provide mechanistic insights into drug action. Although our compound set was small, we found that compounds with similar mechanisms of action and structural analogs clustered together in morphological space, demonstrating the method's ability to infer mechanistic relationships (Fig. 5.3).

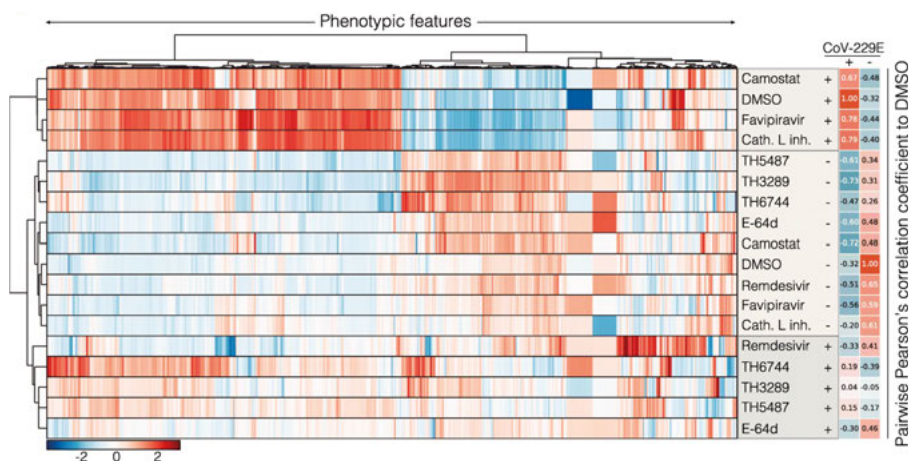


Figure 5.3. Unsupervised hierarchical clustering of morphological profiles for non-toxic compounds. The dendrogram shows a large cluster containing effective antivirals together with non-infected controls, and a separate cluster of ineffective antivirals grouping with infected controls. Structural analogs (TH3289, TH6477, TH5487) and protease inhibitors (Camostat and E-64d) cluster together. Pearson correlation coefficients are shown relative to infected and non-infected controls.

In summary, we present a novel approach for screening antiviral compounds that (1) identifies virus-induced phenotypes from cell morphology, (2) enables screening for compounds that reverse these virus-induced phenotypes, and (3) captures detailed host-cell responses, providing context on cell health and mechanistic insights into drug action through morphological similarity. The platform can be used without prior knowledge of antiviral mechanisms of the compounds, and has the potential to be used to study a wide range of cell systems and viral pathogens.

5.2 Paper II: A systematic host-centric approach to identifying repurposed drugs for viral infections

Building on the methodology introduced in paper I, here we expanded it for screening a large library of repurposable drugs against SARS-CoV-2. We compared the results to conventional antiviral screening approaches, validated the hits in a second cell line, and implemented a counter-screen for phospholipidosis, a frequent confounder in antiviral screens.

Identification of Repurposable Drug Candidates

We first confirmed that the method could be applied to new cell lines and viruses, demonstrating that SARS-CoV-2 induced prominent morphological

changes in both African green monkey Vero E6 cells and human lung A549-ACE2 cells. We then systematically screened a drug repurposing library comprising 5,275 repurposable drugs using four complementary readouts: viability assessment across five cell lines, cytopathic effect (CPE) inhibition, morphological rescue, and viral infectivity based on antibody staining. We identified 150 compounds that rescued the virus-induced phenotype, defined by a "morphology score" measuring the distance to non-infected controls. Approximately two-thirds of the hits identified through morphological profiling overlapped with those detected by antibody-based and CPE assays, whereas 52 hits were uniquely identified by Cell Painting (Fig. 5.4), demonstrating the complementary nature of these screening approaches.

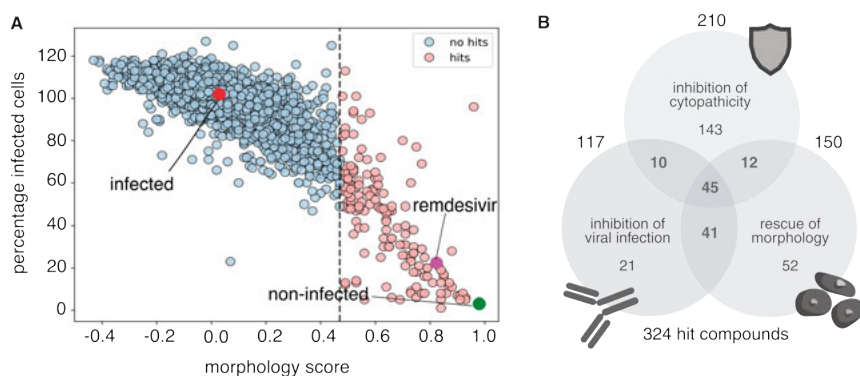


Figure 5.4. (A) Scatter plot showing compound effects on infection rate versus morphology score, with hits restoring normal cell morphology highlighted in red. (B) Venn diagram comparing compound hits that inhibit cytopathic effects, suppress viral infection, and rescue cell morphology, resulting in 324 identified hit compounds.

Hit Validation and Target Pathway Analysis

To validate the hits from the three assays, we screened 324 compounds in human lung A549-ACE2 cells. We implemented a counter-screen for phospholipidosis, a common confounder in antiviral screens, caused by compounds with non-specific *in vitro* antiviral activity rather than target-based activity relevant *in vivo* [133]. We then prioritized hits with favorable properties for advancement as antiviral drugs by defining a target product profile (TPP) that considers factors such as route of administration and clinical phase. Furthermore, Gene Ontology enrichment analysis of the 649 annotated drug targets revealed that the validated compounds target host processes frequently hijacked by viruses, including innate immune responses, kinase signaling pathways, peptidyl-tyrosine modification, and MAPK signaling. Among the top hits we found multiple candidates previously reported as broad-spectrum antivirals across different virus families, including sunitinib, emetine, and cy-

cloheximide, as well as novel antiviral candidates such as serdemetan, BMS-566419, and metixene.

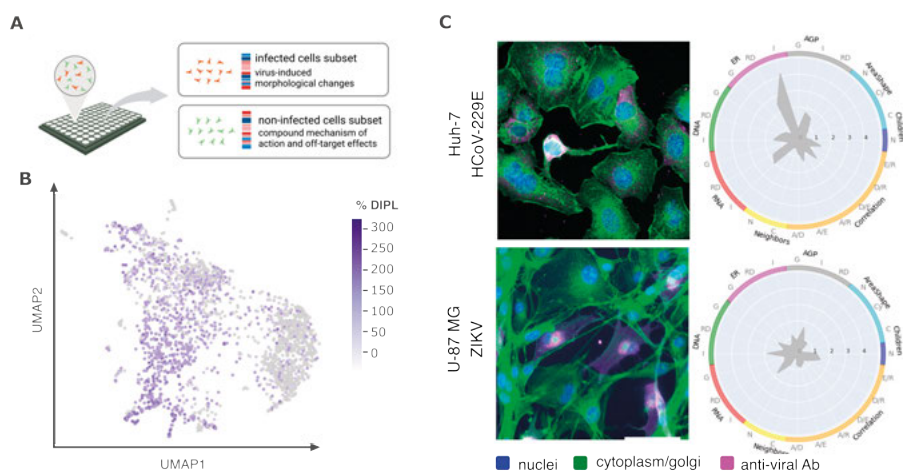


Figure 5.5. (A) Schematic illustrating separation of infected and non-infected cell subsets to capture virus-induced and compound-specific morphological changes. (B) UMAP projection of non-infected morphological profiles colored by phospholipidosis levels as measured by the DIPL assay. (C) Representative images and corresponding radar plots highlighting distinct morphological profiles induced by HCoV-229E infection in Huh-7 cells and ZIKV infection in U-87 MG cells.

Detection of Off-target Compounds

By stratifying the data based on viral infection status, we analyzed the non-infected subpopulations to study compound effects independently of viral infection. Consistent with our previous study, compounds sharing the same mechanism of action tended to cluster together. Furthermore, we show that morphological profiles of cells exposed to DIPL inducers exhibit high similarity, and that resemblance to well-known phospholipidosis inducers, such as fluoxetine hydrochloride, may indicate this off-target effect (Fig. 5.5B). Finally, we demonstrate that the approach generalizes across seven cell lines and three viruses (229E, SARS-CoV-2, and Zika virus), all producing a detectable morphological phenotype, confirming its broad applicability (Fig. 5.5C).

All in all, this work establishes the antiviral phenomics platform as a scalable and robust tool for host-directed antiviral drug screening, capable of identifying promising antiviral candidates, including broad-spectrum host-targeting hits, while simultaneously revealing insights into their mechanisms of action, and detecting off-target liabilities.

5.3 Paper III: Morphological profiling of environmental chemicals enables efficient and untargeted exploration of combination effects

In our environment, chemicals occur in complex mixtures, yet risk assessment traditionally focuses on chemicals one at a time. One reason for this limitation is that the huge combinatorial space makes screening using conventional methods infeasible. Here, we assess Cell Painting as a method to efficiently screen chemical combinations for their effects on cells.

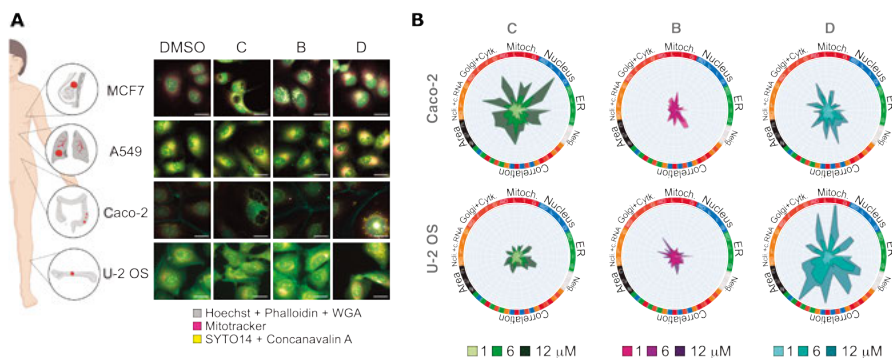


Figure 5.6. Cell Painting image crops for the four cell lines used in this study after exposure to chemicals at 6 μM for 24 h. Radar plots showing the distinct morphological responses across two of the cell lines.

Characterizing Dose-Dependent and Cell-Specific Responses

We selected three chemicals commonly found in everyday products and environments: Cetyltrimethylammonium bromide (C), Bisphenol A (B), and Dibutyltin dilaurate (D). We performed Cell Painting on four biologically diverse cell lines (MCF7, A549, Caco-2, and U-2 OS). We first characterized the morphological effects of single chemical exposures. This analysis revealed that exposure to each of the individual compounds induced dose-dependent morphological effects that were distinct across the four cell lines, both in terms of magnitude and in the specific feature groups and organelles affected (Fig. 5.6).

Co-exposure with BPA exacerbates Morphological Effects

An interesting finding was that BPA, which alone did not cause notable morphological alterations, markedly enhanced cellular responses when combined with other chemicals. In particular, co-exposure of CTAB and BPA led to a

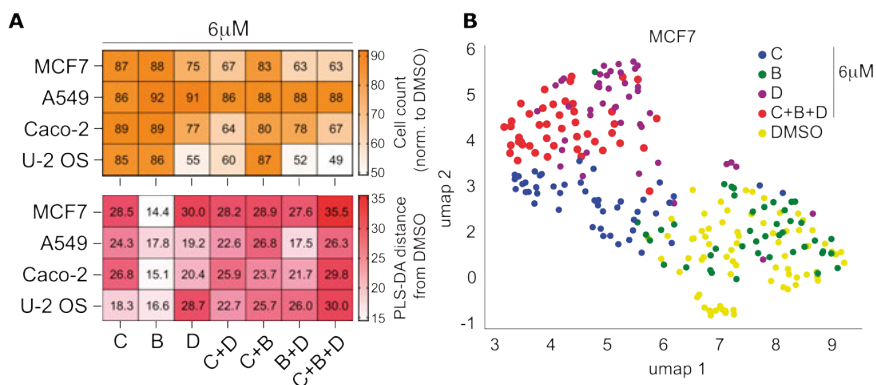


Figure 5.7. (A) Comparison of cell count and PLS-DA distance across single and combined exposures. (B) UMAP visualizing the morphological profiles for MCF7 cells for single or triple exposure.

pronounced exacerbation of morphological effects and induced distinct cellular features not observed under single exposures, including alterations in endoplasmic reticulum and mitochondrial intensity.

In summary, we demonstrate that morphological profiling offers a scalable, cost-effective, and information-rich approach to assess combination effects of chemicals, enabling systematic evaluation of chemical mixtures and advancing our understanding of their health risks.

5.4 Paper IV: Assessment of environmental chemicals through cell-based morphological profiling

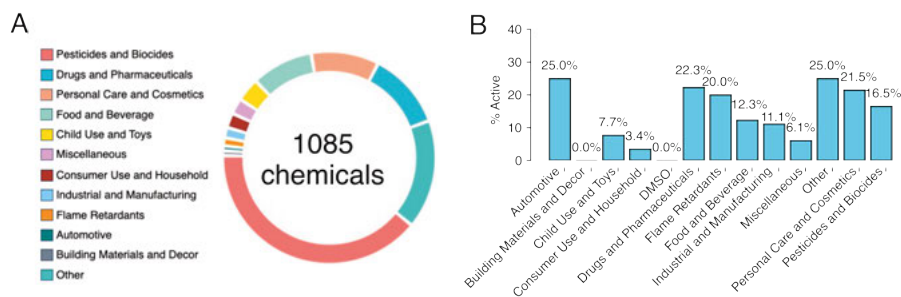


Figure 5.8. (A) Overview of the chemical library showing the distribution of different usage classes of the chemicals included in the study. (B) percentage of significant actives across different chemical classes on U-2 OS cells.

Humans are exposed daily to hundreds of chemicals through food, consumer products, and the environment, many of which remain poorly characterized with respect to their potential adverse health effects. In this study, we established a library of diverse environmental chemicals. We screened the library across five human cell lines to study bioactivity and infer potential mechanisms of action.

Assessment of Bioactivity

We established a library of 1,085 compounds representing diverse use classes (Fig. 5.8A). The entire library was screened on U-2 OS cells (Fig. 5.8B) at 0.6, 5.0 and 50.0 μM . Compounds from the automotive, drug and pharmaceutical, and flame-retardant classes showed a high percentage of significant active responses, as determined by the mean average precision (mAP) scores [76]. In contrast, chemicals belonging to the building materials and consumer-use categories exhibited low activity at the tested doses.

Comparison of Structural and Morphological Similarity

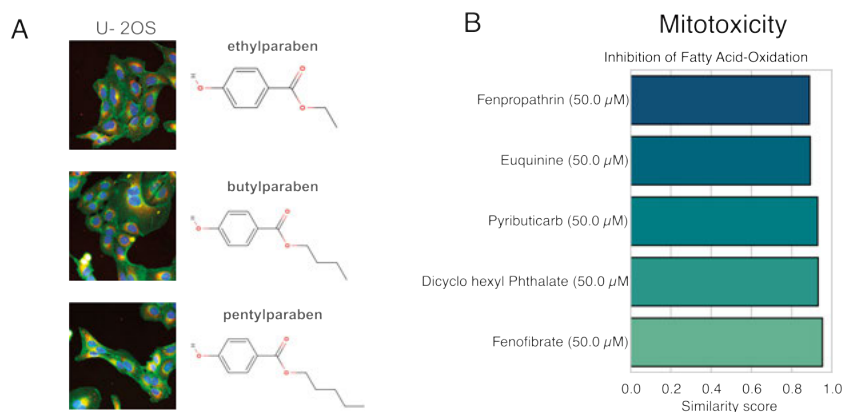


Figure 5.9. (A) Comparison of morphological effects induced by three structurally similar parabens: ethylparaben, butylparaben, and pentylparaben on U-2 OS cells (B) Morphological similarity of top five test compounds to the reference mitotoxicity profile

We next compared structural and morphological similarity for a subset of (potential) endocrine-disrupting chemicals. In some cases, structurally similar compounds exhibited large differences in biological activity in the Cell Painting assay. This was, for instance, the case for a group of parabens that differed only in their alkyl chain length. Ethylparaben induced only subtle morphological effects, whereas butyl- and pentylparaben caused substantial

morphological changes despite their close structural resemblance (Fig. 5.9A). This example illustrates how Cell Painting could be used to identify cases of "activity cliffs", where structurally similar compounds exhibit very different biological effects that may be missed using structure-based read-across approaches.

Elucidating Mechanisms

To identify potential mechanisms of action, morphological profiles were compared to reference MOA signatures using cosine similarity, exemplifying how this approach can infer mechanisms for environmental chemicals. As shown in Figure 5.9B, several industrial and environmental chemicals, including phthalates and pesticides, clustered with mitotoxic references.

This study presents one of the largest morphological profiling screens focusing on environmental chemicals. By integrating phenotypic signatures with structural and reference data, we can identify bioactive chemicals, uncover mechanisms of action, and provide information-rich biological data that complements structure-based predictions.

6. Discussion

When I started my PhD in early 2020, I set out to study what is often called the 'silent pandemic' of chemical exposures and their effects on human health. Only a month later, SARS-CoV-2 drastically changed this, leading us to adjust the project aims to focus on antiviral screening. This unforeseen event allowed me to apply morphological profiling in two distinct fields: drug screening and environmental toxicology. In this chapter, I reflect on what I have learned over the past five years and discuss how the results of my work contribute to both antiviral drug discovery and chemical safety assessment.

Antiviral Drug Screening

When SARS-CoV-2 emerged and disrupted global health systems, we recognized an opportunity to contribute to antiviral drug discovery. In collaboration with the Karolinska Institute, we evaluated whether morphological profiling could enhance phenotypic screening by quantifying virus-induced host-cell changes. In papers I and II, we demonstrated that morphological signatures reliably capture infection-related phenotypes, are robust across cell lines and viruses, and scale readily to screen thousands of compounds. We found that compounds with similar mechanisms or chemical structure clustered in morphological feature space, which is valuable for elucidating mechanisms of action for novel antivirals or identifying off-target effects. These findings establish that morphological profiling offers key advantages over conventional assays by simultaneously revealing infection levels, host-cell states, and insights into compound mechanisms and off-target effects.

We believe the established approach has great potential to play a role in future antiviral screening campaigns. In fact, recent studies have been conducted using similar approaches combining morphology and antibodies to screen for antiviral agents against SARS-CoV, [134, 135], and Flaviviruses [136]. Beyond viruses, the same approach could be applied to other infectious diseases. Recent work has used morphology to study human pathways required for malaria parasite development [137, 138]. Nevertheless, applications to bacterial infections, antibiotic or bacteriophage screening, and other intracellular parasites remain largely unexplored and represent promising directions for future research.

Chemical Hazard Evaluation

The second aim of this thesis focuses on applying morphological profiling to study the effects of environmental chemicals on cells. Whereas multiple studies have been conducted studying environmental chemicals, they typically only include a small number of chemicals. Previous large-scale efforts by the U.S. EPA (screening 462 and 1,201 chemicals from the ToxCast library) have given valuable proof of the potential of Cell Painting in screening-level hazard assessment but are restricted to a single cell line (U-2 OS), do not include benchmarking compounds with known mechanisms of action, and raw data is not publicly accessible [54, 22].

In paper IV, we addressed these limitations by establishing a diverse library of 900 environmental chemicals, screened across multiple cell lines. We show that inclusion of additional cell lines better picks up endocrine-disrupting effects, and show examples of how morphology data can be used to identify instances where structurally similar compounds induce very distinct biological effects. By openly sharing the resulting data, we envision filling an important gap, facilitating the evaluation of understudied environmental chemicals, and advancing the development of Cell Painting as a New Approach Methodology (NAM).

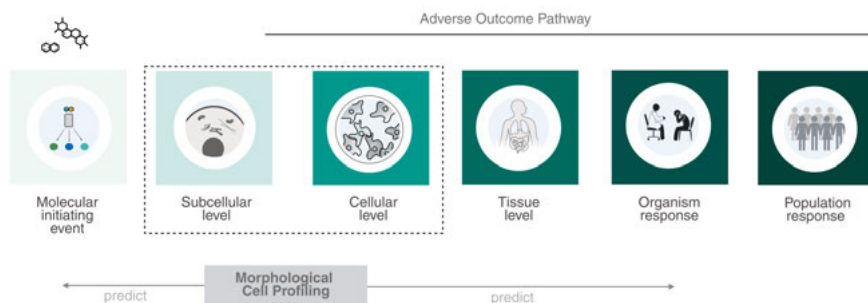


Figure 6.1. Schematic illustration of how morphological cell profiling can be integrated within the Adverse Outcome Pathway (AOP) framework. By capturing sub-cellular and cellular responses, morphological profiling can provide mechanistic links between molecular initiating events and higher-level tissue, organism, and population outcomes, supporting its potential role in Next-Generation Risk Assessment (NGRA).

In the broader context, the potential of Cell Painting within Next-Generation Risk Assessment (NGRA) is only beginning to emerge. While the assay has demonstrated utility for estimating potency and elucidating mechanisms of action [22], its regulatory adoption requires greater robustness and a clear toxicological context (e.g., relevant cell models) [139]. Embedding morphological data within the Adverse Outcome Pathway (AOP) framework [140] offers a promising route to achieve this. Because AOPs organize toxicity mechanisms from molecular initiating events to adverse outcomes, linking morphological features to specific key events could mechanistically anchor phenotypic

changes observed in Cell Painting. This integration could enhance the interpretability of the high-dimensional data and support the use of Cell Painting as a mechanistically informative component within NGRA (Figure 6.1).

Combination Effects

The third aim of this thesis was to explore the use of Cell Painting to study the combination effects of chemicals. In paper III, we demonstrated that Cell Painting can reveal combination-specific morphological changes that do not arise from individual exposures. For example, Bisphenol A produced only subtle effects alone, but exacerbated morphological effects when combined with two other chemicals. This aligns with the "something-from-nothing" phenomenon often reported for endocrine-disrupting chemicals [114].

Although this was a proof-of-concept study using a small set of chemicals, it demonstrates that morphological profiling can capture mixture effects. Recent studies using Cell Painting for PFAS mixtures [123, 124] show an interesting direction in studying more human-exposure relevant combinations, but all remain small-scale. Future studies should increase throughput, include compounds with diverse mechanisms, and apply computational approaches to discern additive, synergistic, or antagonistic interactions. One interesting avenue for future research would be to directly study environmental samples using Cell Painting, such as soil or water extracts. Morphological profiling could be integrated with so-called effect-based frameworks, which iteratively fractionate environmental samples to identify causative chemicals and synergistic effects in an unbiased way [101].

Bridging Pharmacology and Toxicology

In this thesis, I have worked at the interface of pharmacology and environmental toxicology. While these fields pursue different goals, they share fundamental principles, concepts, and screening methodologies. These fields can mutually benefit from methodological and conceptual transfer: environmental toxicology provides insights into low-dose chronic exposures and mixture effects that can inform drug safety assessment and off-target prediction. Conversely, pharmacology's focus on mechanistic understanding and structure-activity relationships can enhance toxicological hazard prediction and risk assessment. I demonstrate this in paper IV, where I leveraged well-annotated drugs with known mechanisms to predict the biological mechanisms of environmental chemicals through morphological similarity.

Despite this potential, applying methods across fields is not always straightforward. Pharmacological methods are optimized for drug-like molecules with well-defined targets, while industrial chemicals are not designed for biological activity in humans and often exhibit low bioactivity, unknown molecular tar-

gets, and polypharmacology or general cellular stress responses [22]. Moreover, they can be volatile, poorly soluble, and structurally diverse, making them challenging for high-throughput screening and placing them outside the applicability domains of typical predictive models.

Nonetheless, both fields face a common methodological challenge: most assays rely on single-target readouts that are expensive, information-poor, and difficult to integrate, capturing only a narrow slice of cellular response. In toxicology, examples include engineered receptor cell lines using luciferase reporters (e.g., for estrogen receptor binding). In drug screening, enzyme activity assays (e.g., for kinase activation) are common. Morphological profiling addresses this limitation by providing a single assay that interrogates multiple biological endpoints simultaneously, generating rich, multidimensional cellular representations. This "one-for-all" approach offers a shared methodological framework with substantial potential for advancing both toxicology and pharmacology.

Experimental and Methodological Advances

Over the course of this PhD project, our laboratory transitioned from manually executed Cell Painting experiments to a fully automated platform. This transformation involved scaling from manual 96-well assays to a robotic system capable of screening up to sixty 384-well plates per day. A substantial part of this work focused on optimizing experimental parameters to ensure high levels of reproducibility and avoiding batch effects, which present a major challenge in high-content imaging [71]. Sources of variability, such as cell seeding density, wash settings, compound concentration, and reagent concentration and lot effects, all influence data quality and comparability [129]. Automation and standardization not only improve within-laboratory reproducibility but also hold substantial potential for enabling cross-laboratory comparisons. This could facilitate integration with large-scale reference datasets such as those from the JUMP consortium containing 136,000 chemical and genetic perturbations, thereby opening up many new opportunities for how the data can be used beyond the immediate experimental context [32, 129].

Beyond optimizing the core assay, this work introduced a novel adaptation of Cell Painting by integrating antibody-based markers into the staining panel. Besides antiviral research, this hybrid approach provides a general strategy to extend the assay's capabilities. It could, for example, be used to incorporate markers for cells expressing specific proteins of interest, exhibiting disease-relevant phenotypes, or to enable co-culture experiments where antibody markers stratify different cell types within the same well. Such integration provides ground-truth labels that will allow the development of predictive models linking cellular morphology to function at the single-cell level. To overcome the limitation of sacrificing one fluorescence channel, future imple-

mentations could employ iterative stainings, which would further enhance the assay's versatility and information content [69].

Confounders and Limitations

Several confounding factors must be considered when interpreting Cell Painting results. The choice of cell model is a major determinant of the interpretability of Cell Painting data. The molecular target may be absent in the selected cell line, or expressed but lacking downstream signaling components required for a response. For instance, when studying endocrine disruption, relevant receptors must be expressed to be able to detect these effects [22]. Some compounds require metabolic activation to form reactive metabolites, which cannot occur in metabolically inactive cells and may thus necessitate more advanced models [141]. While cancer cell lines might be limited in their ability to represent normal cellular physiology, much of the current large-scale morphological profiling data has been generated in simple, robust models such as U-2 OS or A549. These non-specialized cell lines have consistently demonstrated their capacity to capture a broad range of biologically meaningful perturbations and yield valuable insights across diverse applications [34]. Selecting an appropriate cell model, therefore, requires balancing physiological relevance, practicality, and interpretability, while recognizing that even simple systems can provide powerful screening platforms for initial discovery and hypothesis generation.

In addition to biological considerations, technical factors can also confound morphological profiling experiments. Compound auto-fluorescence can interfere with image-based measurements and lead to false morphological signals [24]. Moreover, neglecting pharmacokinetic factors and the physicochemical properties of chemicals can significantly impact experimental outcomes. For instance, physicochemical properties that influence compound adsorption to multi-well plates, protein binding, insolubility, and instability in media can cause substantial deviations between nominal and effective concentrations in the assay. Additionally, the lack of compound resupply may lower effective concentrations over time. To mitigate these issues, incorporating chemical distribution and pharmacokinetic models, as well as improving the standardization of experimental conditions such as culture media composition, could improve the physiological relevance of the acquired data [142, 4, 143].

Methodological Perspectives and Complementary Approaches

While Cell Painting has proven powerful in capturing diverse cellular mechanisms and toxicological responses, it also has inherent limitations. Unlike molecular omics technologies such as transcriptomics, proteomics, and metabolomics, it provides only indirect insight into underlying biological path-

ways. Interpretations rely mostly on reference data, which may not always be available, and currently it is hard to compare across laboratories due to batch-effect [129]. Moreover, not all biological activity manifests in a change in morphology. Integrating Cell Painting with molecular omics holds great promise. Recent studies show that morphological and molecular readouts capture complementary aspects of cellular responses [7]. Combining Cell Painting with transcriptomics, proteomics, or metabolomics can yield a more holistic understanding of mechanisms driving phenotypes and improve predictions of chemical effects [144, 145, 18, 124, 146, 147, 148]. Such multimodal strategies represent a promising future direction, enabling more accurate profiling of cell states and advancing the prediction of chemical hazards and drug efficacy [147, 148].

7. Future Perspectives

Looking ahead, several promising directions are emerging for morphological profiling in biomedical and toxicological research. Future progress will benefit from integrating more complex biological systems, including dissociated organoids, patient-derived fibroblasts [149, 64, 65], iPSC-derived cells, co-cultures, and tissue-based imaging [150, 151], which can enhance physiological relevance while maintaining scalability. Additionally, incorporating temporal and spatial dimensions through multi-timepoint, live-cell, and 3D imaging strategies [152, 80, 153, 81, 82, 83] may provide more comprehensive insights into cellular processes and dynamic responses. However, these approaches require systematic validation and standardization before routine high-throughput implementation.

Deep learning has reshaped image-based profiling at several levels. Advanced segmentation tools, such as CellPose, have improved accuracy and generalizability across diverse cell types [72], while self-supervised learning models like DINO are opening new possibilities for deriving biologically informative image representations [154]. Beyond image analysis, artificial intelligence-driven "smart microscopy" approaches could eventually guide imaging acquisition in real time [155], while complementary AI-based experimental design methods may accelerate the exploration of vast combinatorial chemical spaces [156, 157]. These innovations mark a shift from simply generating more data toward producing data more intelligently and efficiently.

As data generation becomes increasingly accessible, the next major challenge lies in data integration and interpretation. We need models and frameworks capable of integrating heterogeneous data types to uncover mechanistic insights and enhance predictive modeling. Emerging foundation models provide a promising avenue by enabling automated, multimodal integration across molecular, cellular, and phenotypic levels. These approaches hold great promise for advancing both predictive performance and our understanding of complex biological processes [158].

In conclusion, this thesis has advanced the methodological and practical frontiers of morphological profiling through diverse new applications in antiviral screening, compound combinations, and environmental chemical assessment. Looking forward, the continued evolution of advanced computational methods, data integration, and physiologically relevant models promises to enhance this approach's impact on chemical safety assessment, reduction of animal testing, and pandemic preparedness.

8. Populärvetenskaplig sammanfattning

Varje dag utsätts våra kroppar för en mängd olika påfrestningar. Virus, bakterier, föroreningar och industriella kemikalier interagerar ständigt med oss, ibland på sätt som vi knappt märker, ibland med allvarliga konsekvenser. Att förstå hur dessa stressfaktorer påverkar vår hälsa är en av de stora utmaningarna mänskligheten står inför idag.

På cellnivå lämnar dessa exponeringar synliga fingeravtryck. Vissa virus får närliggande celler att smälta samman till jättelika "superceller" kallade syncytier, medan andra utlöser bildandet av onormala klumpar av viralt material inuti infekterade celler. Kemiska exponeringar lämnar sina egna distinkta spår: vissa tungmetaller skadar mitokondrier och stör cellernas energiproduktion, industriella lösningsmedel kan spräcka cellmembran, och bekämpningsmedel kan orsaka att ovanliga vakuoler ansamlas i cytoplasman. De påfrestningar som en cell har utsatts för avspeglas i dess form och struktur.

För att kunna se dessa mikroskopiska fingeravtryck utvecklades en metod som kallas Cell Painting. Den går ut på att färga in olika delar av cellen med olika ämnen så att de syns tydligt i mikroskop. När cellerna fotograferas i hög upplösning framträder mönster som avslöjar hur de har förändrats. Från bilderna kan datorer sedan räkna ut tusentals mått på cellernas form och struktur. Med hjälp av maskininlärning kan dessa data användas för att upptäcka dolda mönster och likheter mellan olika behandlingar. Utförd i miniaturiserat format med hjälp av robotiserade laboratorier gör den det möjligt att testa tusentals förhållanden samtidigt.

I denna avhandling användes Cell Painting för att studera hur både virus och kemikalier påverkar celler. I de två första studierna tillämpades metoden för att undersöka hur coronavirusinfektion skapar distinkta cellulära mönster och för att screena efter antivirala läkemedel som kan återställa cellerna till ett friskt tillstånd. De cellulära förändringarna avslöjade inte bara hur viruset skadar celler, utan också hur olika substanser påverkar dem, vilket bidrog till att identifiera lovande läkemedelskandidater. De två senare studierna tillämpade Cell Painting på miljökemikalier och undersökte både enskilda substanser och blandningar. Den tredje studien visade att Cell Painting kan upptäcka oväntade kombinationseffekter när kemikalier interagerar, och den fjärde studien använde metoden på en stor uppsättning miljökemikalier för att kartlägga deras cellulära effekter och undersöka hur de kan orsaka skada.

Genom att noggrant observera hur celler reagerar på stress, oavsett om den orsakas av virus eller kemikalier, kan vi få insikter både om de hot vi står inför och om de möjligheter vi har för att skydda människors hälsa.

9. Popular science summary

Every day, our bodies are exposed to a variety of different stressors. Viruses, bacteria, pollutants, and industrial chemicals constantly interact with us, sometimes in ways we barely notice, sometimes with serious consequences. Understanding how these stressors affect our health is one of the great challenges humanity faces today.

At the cellular level, these exposures leave visible fingerprints. Some viruses cause neighboring cells to fuse into giant "supercells" called syncytia, while others trigger the formation of abnormal clumps of viral material inside infected cells. Chemical exposures leave their own distinctive marks: some heavy metals damage mitochondria and disrupt the cells' energy production, industrial solvents can rupture cell membranes, and pesticides can cause unusual vacuoles to accumulate in the cytoplasm. The stresses that a cell has been exposed to are reflected in its shape and structure.

To be able to see these microscopic fingerprints, a method called Cell Painting was developed. It involves staining different parts of the cell with different substances so they are clearly visible under a microscope. When the cells are photographed in high resolution, patterns emerge that reveal how they have changed. From the images, computers can then calculate thousands of measurements of the cells' shape and structure. Using machine learning, this data can be used to discover hidden patterns and similarities between different treatments. Performed in miniaturized format with the help of robotic laboratories, it makes it possible to test thousands of conditions simultaneously.

In this thesis, Cell Painting was used to study how both viruses and chemicals affect cells. In the first two studies, the method was applied to investigate how coronavirus infection creates distinct cellular patterns and to screen for antiviral drugs that can restore cells to a healthy state. The cellular changes revealed not only how the virus damages cells, but also how different compounds modify them, which helped identify promising drug candidates. The last two studies turned to environmental chemicals, analyzing both individual substances and mixtures. One study revealed that Cell Painting can detect unexpected combination effects when chemicals interact, while the other study mapped how a large set of environmental chemicals influences cellular structure and function, offering insights into their potential harmful effects.

By carefully observing how cells respond to stress, whether caused by viruses or chemicals, we can gain insights into both the threats we face and the opportunities we have to protect human health.

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