Lab on a Chip

Engineering cell heterogeneity into organs-on-a-chip

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Abstract:

Organ-on-a-chip development is an application that will benefit from advances in cell heterogeneity characterization because these culture models are intended to mimic *in vivo* microenvironments, which are complex and dynamic. Due in no small part to advances in microfluidic single cell analysis methods, cell-to-cell variability is an increasingly understood feature of physiological tissues, with cell types from as common as 1 out of every 2 cells to as rare as 1 out of every 100,000 cells having important roles in the biochemical and biological makeup of tissues and organs. Variability between neighboring cells can be transient or maintained, and ordered or stochastic. This review covers three areas of well-studied cell heterogeneity that are informative for organ-on-a-chip development efforts: tumors, the lung, and the intestine. Then we look at how recent single cell analysis strategies have enabled better understanding of heterogeneity within *in vitro* and *in vivo* tissues. Finally, we provide a few work-arounds for adapting current on-chip culture methods to better mimic physiological cell heterogeneity including accounting for crucial rare cell types and events.

1. Introduction

How many types and sub-types of cells should be used in an organ-on-a-chip to provide a sufficiently physiological representation of their macroscopic human organ counterpart from a functional perspective? This article addresses the question by looking to recent advances in characterization of cell heterogeneity and cell plasticity and how it impacts tissue and organ function. Incorporation of sufficient cell-to-cell variability in on-chip culture devices is important because lack of it can hamper disease understanding, drug evaluation and cures. To mention cancer as an example, organs-on-a-chip which incorporate a patient's tumor cells, but

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fail to incorporate all the phenotypes, such as excluding the rare but critically important chemotherapy-resistant sub-populations of cells, could fail to predict the clinical result, as a prescribed therapy may not disrupt all diseased cells to the same effect due to heterogeneity within the tumor. Not only will such cells express a phenotype that may render the treatment ineffective in killing them, but such persister cells may then re-configure the entire tumor to be more difficult to destroy after initial therapy.[1]

Just as the makeup of cells in most healthy and diseased human tissues is heterogeneous, so it may need to be in the tools used to study them. Microfluidic devices designed as organs-on-a-chip have gained complexity since early concepts were first conceived, and it is starting to become standard procedure to use tissue-specific primary cells instead of cell lines to create a better on-chip model that will provide useful readouts for researchers and drug developers.[2] Thorough characterization of cell makeup and cell-to-cell variability is important to validate the next generation of devices and systems. Additionally, understanding the frequency of so-called "rare cells" *in vivo* and in a modeled tissue may prove useful for establishing the lower limit in tissue size when designing an *in vitro* device or system. This review discusses the numerous categories of cell-to-cell variability within tissues, a spectrum of which are highlighted in **Figure 1**, ranging from near 1-to-2 ratios of lung epithelial cell sub-types to rare tumorigenic cells within leukocyte tumors. Strategies for characterizing cell type and organ-on-a-chip device variability to confirm that a design adequately mimics *in vivo* cell heterogeneity are also highlighted, as are strategies to achieve more realistic cell heterogeneity within organ-on-a-chip design constraints.

2. Cell-to-cell differences

Before we can think about constructing organ-on-a-chip systems with physiologically-relevant cell heterogeneity, we need to know what degree of heterogeneity there may be *in vivo*. This section scans the cellular landscape from a high-level viewpoint of species-based differences all the way down to a high-resolution analysis of variability that arises within cell types traditionally considered as a single type (see **Figure 2**). This last area of heterogeneity amongst cells traditionally considered to be one type is being mapped out only recently through advances in single cell analysis techniques, including some which use a microfluidic platform.[3–5] The Human Cell Atlas project was launched in 2016 to obtain and catalog

information about every type of cell in the body with the vision of using such a database to improve study of biology and to more quickly translate our knowledge into real treatments.[6] Beyond the inherent biological significance of the topic, these technological and scientific advances highlight the timeliness of engineering cell heterogeneity into organ-on-a-chip development.

2.1 Species-level differences

Many protein-coding genes are conserved among mammals, making it possible to translate biological results from a model species to humans. Yet failures in pre-clinical screening of therapeutics in animal models to translate to the clinic highlight important differences. Recent molecular analysis of functionally important non-coding DNA also reveals that species differences are much larger than appreciated based on previous comparison focused on protein-coding genes.[7] Thus, based on both industry experience and molecular understanding of species differences, human cells are the preferred species. The ability to use primary human cells is one of the recognized advantages of organ-on-a-chip systems over animal models. That said, proof-of-concept studies benefit from comparing animal models with organ-on-a-chip systems comprised of primary animal cells for concept validation.

2.2 Variability between individuals

The *in vitro* workspace is a difficult setting for replicating the genotypic and phenotypic variability of a target human population. Diagnostics such as CYP 450 screens and biomarker testing are currently used by physicians to gather data about patients before prescribing already approved and marketed drugs to prevent adverse reactions and to maximize benefit through personalized treatment. Experimental technologies for pre-clinical population screening are limited, one successful example being the use of liver microsomes, enzymes isolated and pooled from multiple donors, for drug metabolism assays. Organ/human-on-a-chip developers have envisioned generalizable platforms where the device is applicable for screening any number of agents that have relevance at the studied tissue or disease site.[8] Whether such a generalized platform translates to a generalized human is debatable, however. For basic applications, where key gene expression patterns are conserved across the entire population, a generalized device might have efficacy, but variability

among individuals could be accounted for in early-stage efficacy and toxicity studies to increase research and development efficiency.

Whether further person-to-person variability will be ultimately addressed by a chip array with replicates representing known genetic or phenotypic variants remains to be seen.[9] The numbers of unique organ chips required to represent the human population based on the number of pooled donors for typical liver microsome drug metabolism studies would suggest 40-50,[10] or as many as 12,000 based on the number of unique HLA Class I alleles when considering immune responses.[11] Discussions with colleagues and comparison with clinical trial subject numbers suggest an ideal number of distinct organs-on-a-chip to be in the order of thousands constructed using primary human cells from across a broad demographic range in terms of age, sex and ethnicity to capture the human population. Already, a cutting-edge impact breast cancer organoid research article has created a living biobank with over 100 breast cancer organoids generated from over 150 different breast cancer patients.[12] For organ-on-a-chip experiments, it is starting to become important to test devices using cells from multiple donors to determine whether results correlate with the donor's condition or pre-dispositions. On the other hand, for many diseases there is already significant effort to stratify patients into distinct sub-populations based on molecular and cellular phenotypes, and disease severity. These efforts make construction of clinically-relevant patient populations-on-a-chip more feasible and meaningful. For example, rather than constructing a general intestinal cancer *in vitro* model, one can construct a library of tumors cultured *in vitro* from less harmful primary adenomas to the most harmful metastatic carcinomas.[13] It is relevant to note that the National Institutes of Health now requires consideration of biological variables such as age, weight, underlying health conditions, and particularly sex, in grant applications.[14,15]

2.3 Organ-to-organ differences

Before considering human-to-human variability, the organ-on-a-chip field faces considerable challenges even in construction of one specific individual. Micro-physiological system developers interested in linking multiple organ compartments together have already started to explore the feasibility of working with cells from multiple organs and connecting them by a single fluid compartment to enable cross-talk. This

pursuit of a human/body-on-a-chip homeostasis or disease model is exciting, but well-recognized, with many existing reviews on the topic to which interested readers are referred.[8,16–19] The ability to use primary human cells in a physiological microenvironment, with organ-to-organ connectivity is one of the well-regarded advantages of organ-on-a-chip systems and is not explored in depth in this review.

2.4 Cell type heterogeneity

Knowing the cellular makeup of the target organ may be critical for determining how to combine multiple cell types. The human body features a plethora of microenvironments which may be modeled on-chip, each with its own diversity of cellular players. Additionally, each organ contains a diversity of cell types to carry out their function. The human lung serves as a robust example, and a relevant one for organ-on-a-chip development, as many publications have emerged on lung-mimicking devices.[20–23] The lung is a large organ, containing approximately 230 billion cells.[24] The alveolus alone, arguably the minimal functional unit of the lung, contains two epithelial subtypes, macrophages, endothelial cells and fibroblasts, to name several. Among the large airways, small airways and alveoli, researchers have identified at least 40 different cell types that exist within this single organ.[25] Organ-on-a-chip technologies have reached the complexity of utilizing co-culture of multiple cell types in devices and have already reproduced interesting phenomena. A deeper layer of cell heterogeneity exists, however, which must be better characterized to maximize the predictive capabilities of these devices.

2.5 Intra-cell type heterogeneity

The construction of minimal functional units of tissues and organs, such as a lung alveolus or tumor gland in colorectal cancer, is a common goal of the organ-on-a-chip and organoid fields.[9] The underlying assumption is that by creating such a construct and analyzing its function, the response of an entire tissue or organ can be extrapolated as long as the miniature construct contains all the necessary primary human cell types. As our understanding of intra-cell type heterogeneity increases, however, this assumption becomes questioned. No matter how faithfully a minimal functional tissue unit is constructed, could it possibly represent the intra-cell type heterogeneity that exists across an entire organ? Heterogeneity of this kind is only beginning to be understood, but its ramifications are critical for tissue function and disease response. Among

the same cell types within the same *in vivo* tissue bulk, variability may arise from multiple factors, genetic and non-genetic.

A clinically important example is the cell heterogeneity present in colorectal cancer. In studying the "big bang" model of human colorectal tumor growth, investigators analyzed 349 individual tumor glands from 15 colorectal carcinomas and large adenomas.[26] Despite their likely monoclonal origin from a single aberrant colon crypt, the investigators found that tumor heterogeneity arises relatively early and persists throughout the course of the disease. Particularly challenging from the perspective of constructing minimal functional units of colorectal tumors is the finding that aggressive subclones with a high fitness advantage can remain rare, even undetectable, in primary tumors until there is a selective pressure applied onto the tissue such as surgery or chemotherapy. Yet, such rare subclones of tumor cells would need to be represented somehow in a miniature model.

Genetic variability can also arise within the same cell type also in normal physiological processes such as X-chromosome imprinting in female organisms, where one of the chromosome pair is expressed in some cells and the second is expressed in others, seemingly at random, making a mosaic of cells.[27] Genetic variability can also arise through coexistence of uniquely-mutated cell clones, which may have consequences like differential resistance to chemotherapy. There are multiple documented examples of intra-cell type variability not attributed to genetic mutation as well. These include sensitivity to directed apoptosis based on internal protein availability in mammalian cell lines[28] and apparently stochastic variability which, when the cell reaches its restriction point, results in lineage commitment during hematopoiesis.[29] Transcriptional variability in cancer cells, when under drug-induced selective pressure, leads to drug-tolerant persister cell clones.[1,30]

Worth mentioning, for practical reasons, is the implications of *in vitro*-cultured cell properties: passage number, doublings and culture time on intra-cell type heterogeneity. The largest differences exist between primary cells, those directly isolated from a mammalian tissue, and immortalized cell lines. Primary cells will undergo senescence after a high number of doublings or passages whereas immortal cell lines show greater tendency towards continuous proliferation.

Primary cells may exhibit the greatest heterogeneity during *in vitro* culture in early passages, containing many of the physiological phenotypes found within the isolation constraints. Expansion of a cell population is a process itself that selects for individuals that proliferate well on the 2D surface, which leads to some phenotypic drift from the original primary cell population. As cells approach the Hayflick limit for cell doublings, however, and senescence begins to creep in, functional heterogeneity may also arise where metabolism or response to stimulation show greater deviation. This would reflect what has recently been shown *in vivo*: cells isolated from older individuals show greater heterogeneity in their epigenetic signatures and functional capacity compared to cells isolated from younger individuals.[31,32] Limits on culture time and evidence of intrinsic aging for primary cells from older donors has long been known.[33] Hayflick, himself, found that fibroblast cells from an adult lung underwent less than half as many doublings on average as fibroblasts isolated from a fetal lung.[34]

For immortalized cell lines, cell-to-cell uniformity is expected in early stages of culture at lower passage number, particularly if the phenotype is fully differentiated.[35] A hallmark feature of cell lines is high doubling capacity. Nevertheless, use of exceedingly high-passage cell lines is not recommended, due to observance of diverging phenotypes at late culture stages[36] as well as more general phenotypic drift.[37] The process of transfecting cell lines may generate a phenotypically heterogenous population also, unless individual clones are isolated and their progeny are used exclusively.[38] The next sections of this paper will provide instructive examples of documented heterogeneity within a population of cells of a similar type and discuss its implications on organs-on-a-chip experiments.

3. Tumor heterogeneity

Tumors are an important area for studying intra-cell type heterogeneity. Although often arising from the proliferation of a single diseased cell, there is a surprising amount of cellular heterogeneity that is a critical obstacle to developing cures for the disease. Often the tumor bulk is distinguishable from healthy tissue through known biomarkers, but targeted therapies for that biomarker will encounter resistance from rare cells. Circulating tumor cells (CTCs) have been projected as a possible minimally-invasive source of key information for tumor characterization and personalized treatment,[39] but in the case of a heterogeneous

tumor, CTCs may not tell the whole story.[40,41] For more comprehensive reading, multiple reviews of heterogeneity found in the tumor microenvironment are available.[42,43]

The study of cancer on-chip is one clear application where consideration of cell-to-cell variability is paramount. A recent review of cancer-on-a-chip projects can be found by Portillo-Lara and colleagues.[44] Notable classes include devices to simulate metastatic invasion into target tissues,[45,46] extravasation from a tumor site into the vasculature,[47–49] and tumor angiogenesis.[50,51] Cancer-on-a-chip studies often do not consider tumor cell heterogeneity in detail. Clinical and biomedical researchers, however, have actively studied intra-tumoral heterogeneity in tumors of the breast, intestines, skin and brain among others. For researchers interested in specific tumor to recapitulate on-chip we list some representative characterization studies of intra-tumoral heterogeneity with emphasis on rare and stem-like tumorigenic cells (summarized in **Table 1**). Some tumors carry greater heterogeneity than others, and some cellular heterogeneities are easier to engineer than others. For example, if one can isolate and use breast cancer stem cells, much of the tumor heterogeneity found in the disease can be recreated starting from just a few cancer stem cells.[52] Conversely, the heterogeneity of other tumors, such as colorectal cancer, is less organized and potentially harder to re-engineer.[26] For organ-on-a-chip applications where patient tumor samples or biopsies are used to predict outcomes or drug sensitivity, intra-tumoral heterogeneity is a substantial hurdle as it is difficult to ensure a complete sampling of the entire tumor heterogeneity on-chip.

3.1 Breast cancer

Not all breast cancer cells can re-establish a tumor on their own when removed from a primary tumor and seeded elsewhere, even under optimal conditions. The few special tumor cells that can re-create entire tumors are often referred to as cancer stem cells (CSCs). Breast cancer is the first solid tumor for which CSCs were identified.[52] Breast CSCs were first identified as $CD44^{+}CD24^{-/low}$ in about 2.5-5% of tumor cells, while a newer marker, ALDH1, has been identified in breast cancer as well to classify a tumorigenic cell population that was associated with poor clinical response to treatments.[53] The ability to identify and isolate CSCs is important because it potentially means that one can theoretically generate the heterogeneity of a tumor-on-a-chip starting from a single cell. Although one must be careful, as these stem cells can exhibit

stochasticity in their differentiation and drawing out their plasticity potential may be difficult.[54] Genetic variability of breast cancer cells has also been described, suggesting additional challenges for re-engineering the full heterogeneity of a breast tumor.[55,56]

3.2 Intestinal cancer

Colorectal cancer is the second leading cause of cancer death, despite mechanistic understanding of its origin.[57,58] We now know there are several markers for colonic CSCs. CD133, a marker common for CSCs across tissues, was first identified as being the marker represented on tumorigenic cells in the colon – tumorigenic cells existed at only 1 in 5.7×10^4 total tumor cells, but at 1 in 262 CD133-expressing tumor cells.[59,60] Further work has discovered several other stem cell markers. Dalerba and colleagues identified three important markers: EpCAM, CD144 and CD166. Tumorigenic cells either co-expressed EpCAM and CD44 (found on 1 in 18 primary colorectal tumor cells), or co-expressed EpCAM and CD166 (found on 1 in 16 cells).[61] Other discovered stem cell markers include CD29, CD24, Lgr5, and β-catenin.[62] A more recent study utilizing single-cell transcriptional analysis of colonic tumor cells reported the discovery of a system to classify tumor cells by lineage maturity and clinical outcome, headlined by the marker KRT20.[63] In this work, cells with progenitor-stage gene expression profiles ($Lgr5^+$ and $KRT20^-$) corresponded to unfavorable clinical outcomes.

Lgr5 has emerged as the dominant marker for colonic CSCs.[62,64–66] Despite this, the multiple markers for tumorigenic colorectal cells should still be considered for cancer-on-a-chip applications because of the "big bang" model described previously. This is because while $Lgr5^+$ cells dominate, cells expressing the other markers may still also exist. A recent report considered the heterogeneity of patient-derived organoids, as cells were examined for the markers Lgr5 and KRT20. The work found that heterogeneity is maintained, such that the $Lgr5^+$ stem cells were replenished after ablation.[67] Studying diversity of colorectal cancers across patient cases also has had impact. Fujii and colleagues established a colorectal tumor library of 55 samples from 43 patients, engineering tumor organoids that can mimic the heterogeneity of the tumor of origin, including showing how metastasis-sourced cells could undergo metastasis, where primary tumor-sourced cells failed.[13] Obtaining sufficient primary cell biopsies from patients to represent the entire tumor heterogeneity, however,

is a challenge. These two accounts are good examples of the importance of incorporating heterogeneity into *in vitro* models of intestinal cancer.

The architectures, functions, and maintenance of the small intestine are different than the large intestine, however some key elements of colonic tumor heterogeneity and biomarkers of CSCs also apply. Small intestinal epithelium self-renews the most of all human tissues,[68] which makes adenomas of the small intestine difficult to control and malignancy hard to prevent. So far, Dclk1 has been shown to differentiate tumor stem cell populations in the small intestine from normal stem cells. However, both normal stem cells and tumor stem cells have been shown to have multiple biomarkers in common: Lgr5,[64,69,70] Bmi1,[71] and CD133.[72] $Lgr5^+$ adenoma cells have been shown using lineage tracing to compose 5-10% of the adenoma cell population and were crucial in adenoma growth.[70] One point of consideration is that $Bmi1^+$ intestinal stem cells can regenerate the $Lgr5^+$ pool and thus suggest that a hierarchical structure exists and may need to be considered in establishing both intestinal organoids and *in vitro* cancer models (as some $Bmi1^+$ cells may also be cancer stem cells).[73] CSCs of the intestine might also need to be sub-grouped,[74] with multiple CSC subgroups incorporated, if a "big bang"-modeled tumor on-chip is to be replicated.

3.3 Melanoma

Melanoma is another aggressive form of cancer, typically arising from the skin epithelium.[75] One advancement in the understanding of melanoma tumor heterogeneity is the characterization of a melanoma tumorigenic cell.[76,77] Cells expressing ABCB5, which also confers melanoma chemoresistance, were found to initiate tumors in immunocompromised mice, yet present at a rarity as great as 1 in 10^6 tumor cells.[78] However, examination into the protocols and mouse models behind discovery of these rare, tumor-initiating cells found that these cells may exist with greater frequency, as much as 1 in 4 unsorted tumor cells, refuting the rare cancer stem cell paradigm for melanoma.[79] Having melanoma-initiating cells at such a high frequency suggests it would be easier to grow an accurate melanoma on-chip from such cells. Therapeutically, it makes eradication of all melanoma-initiating cells difficult.

Chemo-resistance in melanoma is a classic example where clonal heterogeneity leads to ineffective treatments. A well-studied example is rare secondary genetic mutations which impart resistance in melanoma

cells to chemotherapy targeting the primary melanoma mutation, BRAF V600E,[80,81] but non-genetic, transcriptional mechanisms have been reported as well.[82] Such rare cells can be positively-selected for by factors such as drug treatment and form new resistant cell colonies.

3.4 Glioblastoma

Glioblastoma is one of the most aggressive and difficult-to-treat forms of cancer, largely due to its spatial heterogeneity with niches containing self-renewing tumor cells. Researchers have worked extensively to characterize these cells, and their classification has evolved through time,[83–86] leaving us still uncertain of whether a definitive marker of tumor cell stemness in glioblastoma exists. More early-on, prominin-1 (CD133) was believed to be a critical indicator of cell capacity for self-renewal within the glioblastoma. It was found that human brain tumors contained down to 3.5% cells expressing CD133 on the surface of pilocytic astrocytoma and 6.1% cells in medulloblastoma when sorted with flow cytometry.[83] However, later studies indicate that CD133 is not essential for tumor cell renewal as was studied *ex vivo* in the capability of explanted cells to form neurospheres when the gene PTEN was deficient in the lineage.[87] These experiments have shown that tumor cell proliferation *in vivo* and *in vitro* will differ depending on the presence or absence of these PTEN-deficient, self-renewing cells. Because of these evolving hypotheses about the source of malignant tumor growth, researchers hoping to study glioblastoma on-chip will need to stay current with future reporting and make use of primary tumor cells when possible to account for these tumor cell sub-types found *in vivo*.

3.5 A note on cancer cell lines

The use of cancer cell lines for on-chip experiments is well-recognized among organ-on-a-chip developers, and their usefulness for proof-of-concept testing affords them an important place in the development process. As with all cells, researchers should be well-informed of their characteristics when using them. As stated in section 2.5, cell lines are usually considered to be homogenous populations of terminally-differentiated cells and may be tumor-derived or transformed to be immortalized.[88] There are exceptions, however, which the reader should be made aware of. Multiple mammalian cancer cell lines have been shown to include heterogeneity at the single cell level, including identified sub-populations, often

referred to as "side-populations" showing exclusive colony-forming potential.[89–92] Prostate cancer cell lines, such as PC3, feature a nomenclature for three sub-populations of cells: holoclones, which have high colony-forming potential; paraclones, which rarely proliferate; and meroclones, which have unique morphology with proliferative characteristics in-between.[93,94] Microfluidic-based single-cell analysis devices have successfully distinguished these cell line sub-populations more recently.[95]

For on-chip cancer models, researchers must consider the suitability of a cancer cell line for their research: the surface marker signature for the disease they intend to model, how rapidly the cells proliferate, or the cells' ability to form tumor-mimicking spheroids. The characteristic of cancer cell stemness varies between cell lines, such as with ALDH1 activity in breast cancer cell lines.[96] Single cells from the same breast cancer cell line, including MDA-MB-231 and MDA-MB-468, have been sorted based on ALDH1 activity; positively-selected individuals display higher activity in proliferation, migration and invasion assays.[97] To further increase translational value, however, organ-on-a-chip developers may require incorporation of primary human cells, such as from several patients within the population intended for treatment.[12,13] To replicate the microenvironment that properly suits the use of primary tumor cells may require even further refinement and engineering of the device platform.

4. Physiological heterogeneity

Healthy tissues in the body also contain high levels of intra-cell type heterogeneity that are only recently gaining appreciation through projects such as the Human Cell Atlas. While organs-on-a-chip using multiple cell types are being constructed, none have yet to capture, in a well-characterized manner, the full diversity of cell types or intra-cell type phenotypic variants. In the near future, it is probably unrealistic to expect organs-on-a-chip that represent the full cellular variability of intact organs. However, it is still important to survey the vast field of cellular heterogeneity of target organs so that one can identify the best subset of cells that will enable analysis of the questions of interest (e.g. epithelial cells plus endothelial cells plus neutrophils to study edema).[23] Another point is to appreciate the heterogeneity that exists among cells that we may traditionally have considered as one cell type, such as intestinal epithelial cells, that are comprised of stem cells and a variety of differentiated sub-lineages with unique features and functions. How then, can

we promote such physiologic intra-cell type heterogeneity in organ-on-a-chip systems? This section discusses two organs in which a significant number of efforts to recapitulate function on-chip have been published: the lung and intestine. As stated previously, the lung microenvironment is a classic organ-on-a-chip target, but the intestine also distinguishes itself in the amount of attention given to recapitulating the microenvironment as a heterogeneous 3-dimensional (3D) organoid.

4.1 The lung

One of the primary difficulties in creating a lung-on-a-chip that accurately models the human lung is that there are at least 40 cell types to consider.[25] The list is not exhaustive as new cell types and even new lung functions, such as platelet biogenesis, have been discovered in recent years.[98] Some cell types may be present or missing depending on factors such as age, sex, or disease. Nevertheless, increasing heterogeneity among lung cells on-a-chip remains seen as a crucial milestone for advancing these devices into more prominent drug discovery roles.[2] As such, rational selection of heterogeneous cells, rare and common, is crucial to establishing an accurate and predictive *in vitro* lung.

Compared to the 40 types of cells and significant intra-cell type heterogeneity characterized in the human lung, on-chip models have incorporated as few as a single cell type derived from a cell line and generally no more than three cell types (epithelial, endothelial and occasionally a circulating immune cell). Suppose we were to narrow down cells from the list of 40 to make a relatively simple lung-on-a-chip. How might we construct a lung epithelium on-chip that would better represent physiological cellular heterogeneity? Lung epithelial cells, endothelial cells and macrophages are the most common cell types.[24] Epithelial cells in the airway consist of several cell types, such as goblet and club cells, which are not characterized in most models, with an exception.[99] A recent study using single cell RNA sequencing (scRNA-seq) to profile epithelial cells in the developing lung identified specific progenitors that may provide insight into selecting primary cell progenitors which are capable of producing all the necessary epithelial lineages for a lung-on-a-chip.[100] Increased cellular diversity has been shown *in vitro* by initiating cultures with multipotent stem cells in an organoid platform. With careful attention to engineering the microenvironment of the developing organoid, Miller and colleagues identified multiple lineages including goblet cells, club cells, neuroendocrine

cells and both types of alveolar epithelial cells derived from the original stem cell population.[101] For on-chip models of lung bronchi, submucosal glands may also be useful to include for infection and inflammation research. These glands produce most of the mucus in the airway, which protects the airway epithelium from microbes.[102] The gland is divided into approximately 60 percent serous cells, which secrete immunoglobulins, and 40 percent mucus cells. Incorporation of such a specialized structure in a lung-on-a-chip could prove useful for specialized models of epithelial defense and pathogen clearance.

What may be of greatest interest is the inclusion of immune cells, even though some may be harder to incorporate into a lung-on-a-chip at the correct numbers. The airways are a major entry point for many pathogens, causing sicknesses like influenza and pneumonia, which bear significant adult and child morbidity and mortality.[103] As such, immune system presence in the lung is important and should be examined. At any given moment, many types of immune cells reside within the lung (**Figure 3A**). Many remain on a more permanent basis, like alveolar macrophages, which exist to clean away microscopic debris that is continuously inhaled. Intra-epithelial lymphocytes also reside more permanently within the alveolar microenvironment, probing for antigens, which may be presented from resident dendritic cells.

Macrophages are one of the most common immune cell types in the lung, comprising around 9% of the total lung by cell number.[24] Resident alveolar macrophages (AMs) can be detected in the lungs within a few days after birth, and they have the ability to self-renew without contribution from bone marrow-derived monocytes.[104,105] Their primary role is to clear pathogens and debris that have infiltrated the airway and reached the alveolus. Because of their numbers and presence in the alveoli throughout human life, AMs may be critical for *in vitro* models of lung epithelia and the alveolus in general, regardless of whether pathogen and cell debris clearance is the object of study. The reader can be referred to a 2014 review for a more detailed insight into AM function, in particular, their relationship with lung epithelial cells.[106]

Other key lung resident cell types include intra-epithelial lymphocytes (IELs) and lymphocytes found in bronchus-associated lymphoid tissue (BALT). Lymphocytes are present in similar numbers; they make up approximately ten billion cells, or 4% of the total lung cell population in healthy adults.[107] IELs are a distinct class of T cells that reside in epithelial layers of tissues and initiate adaptive immune responses against

pathogens. While originally discovered in the small intestine,[108] investigation into the distinct subtypes of intestinal IELs spurred research into bronchial IELs. Bronchial IELs are nearly 99.5% αβ T cells.[109] A far rarer population of T cells in the lung, γδ IELs have yet to reveal a clear role. However, these cells are responsible in the intestinal epithelium, for killing epithelial cells under high stress by recognizing MHC-like stress marker molecules MICA and MICB.[110] Should this role be also observed in the bronchial epithelium, then presence of γδ IELs may be necessary to promote healthy formation of a lung organ culture. Other reasons to include γδ IELs in a lung-on-a-chip are to study their roles in specific diseases like pulmonary fibrosis, autoimmune diseases such as systemic scleroderma, and lung cancer.[111]

While the existence of BALT in healthy human upper bronchial tissues remains a controversial topic, evidence has suggested the existence of infection-induced (antigen-dependent) BALT (iBALT).[112,113] The induced lymphoid tissue contains follicles for B and T cell development, and these cells have been shown to mediate a response to airway influenza infection.[114] Additionally, iBALT could be found in the lungs of mice even when not deliberately infected, suggesting that lymphoid tissues in the lung are continually present in response to opportunistic pathogens. In the context of developing lung-on-a-chip models, an immune component such as iBALT may be needed more often than presumed, as the presence of lymphoid tissue may be more critical for development and normal homeostasis than thought (i.e., not just for studying viral infections, but preventing them).[114]

An important feature of the immune system is its dynamic nature. Neutrophils, the most common leukocyte in the body, do not normally reside in lung tissue, but may extravasate into the interstitium and adopt a new defense-focused phenotype.[115] The healthy lung may have relatively few neutrophils where as an injured or diseased lung may contain many. A lung-on-a-chip microfluidic platform could be ideal for incorporating the dynamic features of the immune into *in vitro* models in ways not possible with conventional static cultures. In a simplified model of immune response to airway epithelial injury neutrophils may be modeled undergoing chemotaxis and extravasation at a wound site (**Figure 3B**). The model could be easily made more complex with the addition of the resident immune system as well to study inflammation onset and healing.

To study the roles of rare cell types and sub-types within the lung would require a more involved experimental design to achieve the correct scaling. While to study alveolar macrophages and a mixed lymphocyte population could be feasible with a miniaturized lung model, studying the adaptive immune system with a reduction in scale would be more difficult , especially to study a rare lymphocyte subset like γδ T cells, which comprise approximately 1 out of every 100-200 T cells, or B cells, which are far outnumbered by their T cell counterparts in the bronchio-alveolar epithelium.[116] Such a case might present a limitation of organ miniaturization.

To adapt this diverse microenvironment on-chip, researchers may choose to consider the resident immune system and dynamic immune system separately or together (**Figure 3B**). A chip with a resident immune system consisting of macrophages, dendritic cells and lymphocytes would support a more generalized approach for screening differential drug effects on the immune system versus the lung epithelium and endothelium, or the interplay between those two major lung components.

4.2 The intestine

The epithelium of the intestine is a dynamic microenvironment with continuous self-renewal.[117] For studying on-chip, it is important to consider the epithelial cell types, which have been the subject of intensive research.[118] In the intestinal crypt, antimicrobial peptide-secreting Paneth cells are most plentiful, and reside next to $Lgr5^+$ stem cells from which they derive. $Lgr5^+$ stem cells also give rise to cell types that exist in the villi: mucus-secreting goblet cells, absorptive enterocytes, hormone-secreting enteroendocrine cells, and antigen-sampling microfold (M) cells.[119] Crypt and villi structures containing these epithelial sub-types can be formed *in vitro* from an $Lgr5^+$ stem cell population without any stroma or mesenchyme present, making construction of intestine on-chip or 3D-cultured epithelial tissue (enteroid) feasible.

One limitation of such an epithelial model, however, is it fails to contain other cells found in the intestine, such as those of the intestinal endothelium or the immune system. Co-cultures including immune cells have yet to make the jump to 3D platforms but a 2D example has appeared recently. Noel and colleagues successfully co-cultured macrophages amongst a 3D culture-derived intestine epithelial monolayer, enabling them to show how the addition of immune cells altered the microenvironment, which better

modeled gut physiology through improved monolayer integrity and replication of immune response to a bacterial pathogen.[120]

For a culture model incorporating the immune system into the intestinal microenvironment, including Peyer's patches may be a good start. Peyer's patches are an important secondary lymphoid tissue in the gut epithelium (analogous to BALT discussed in the previous section on lung) responsible for collecting antigen from the intestinal lumen and enabling immune responses to invading pathogens.[121] Importantly, B cells that mature in Peyer's patches secrete IgA at a level above all other tissues to enhance protection of the intestine epithelial layer.[122] Since Peyer's patches are a significant immune component in the intestinal epithelium, it is something that may need to be included in future models, especially in future cases should a gut microbiome be incorporated as well.

Technically distinct from all-epithelial enteroids are human intestinal organoids (HIOs) which contain epithelial and mesenchymal cells.[123] Derived from induced pluripotent stem cells and cultured in a carefully-controlled environment of biochemical and matrix signals, HIOs also mature to contain many epithelial cell types: Paneth cells and $Lgr5^+$ stem cells in the crypt, goblet cells, enterocytes, and enteroendocrine cells in the villus.[124–126] To use HIO-cultured cells in an intestinal chip presents an additional challenge in comparison to enteroids, as resident mesenchymal cells impede the formation of a continuous epithelial monolayer.[127] EpCAM-positive selection of epithelial cells using fluorescent-activated cell sorting (FACS) circumvents this problem. Using enteroid or organoid-derived cells in organ-on-a-chip micro-channels could enable greater cell-to-cell variability that better-approximates the *in vivo* condition. These successes have inspired a more general work-around for engineering cell heterogeneity on-chip to be discussed later.

5. Strategies for assessing heterogeneity

The increasing recent availability of tools to assess cellular heterogeneity makes this topic particularly timely to consider and address now in organs-on-a-chip systems.[6] Here, we discuss three illustrative examples of different strategies for measurement and analysis as depicted in **Figure 4**. Selection of appropriate techniques and computational analysis methods and further technique development will be important for

obtaining useful information about cell makeup to evaluate the function and physiological relevance of miniaturized organ-on-a-chip model.

5.1 Mapping lineage commitment using mass cytometry

Mass cytometry is an emerging technology for single cell analysis. Its predecessor, flow cytometry, is useful for fluorescently tagging several protein markers simultaneously, typically anywhere from 1 to 10 markers with different fluorophores, depending on the instrument used and user protocol. More recently, mass cytometry was developed to increase the size of the parameter panel to over 30 markers, though with lower throughput.[128] When initially developed, mass cytometry used over 30 markers to successfully categorize a bone marrow cell population into 29 distinct cell types (including detailing 3 platelet types, 5 T cell types and 6 B cell types). With greater numbers of markers, conventional flow cytometry data analysis methods become too limiting and advanced computational methods must be employed to analyze and visualize data. Setty and colleagues used mass cytometry to gather proteomic data of maturing T cells from the thymus then developed an algorithm, which they called "Wishbone", to determine a cell lineage commitment map of T cell progression through the selection of CD4 and CD8 lineages as well as to identify the correct lineages and multiple bifurcations in myeloid cell lineage commitment.[129] These examples are interesting in demonstrating the ability to not only categorize cellular heterogeneity, but in also delineating relationships between the different cell sub-populations identified.

5.2 Identifying rare subtypes of intestinal cells in organoids using scRNA-seq and RaceID

Single cell sequencing methods along with associated bioinformatics tools, such as principle component analysis and clustering are becoming more widely used. 3D organoid cultures, which have also been shown in multiple accounts to serve as important models of cell differentiation and heterogeneity, such as the brain and liver have been analyzed using single cell sequencing methods.[130,131] ScRNA-seq can theoretically detect all actively transcribed mRNA. When analyzing multiple sub-populations, including rare cells, this method provides the most comprehensive analysis of gene expression. Because scRNA-seq experiments provide so much data, the challenge commonly arises at the stage of converting the data into biological insights and understanding.

One relevant example of overcoming this challenge as applied to determining cell heterogeneity is the development of RaceID by Grün and colleagues. This tool was developed to use scRNA-seq data to identify rare cell types and corresponding markers in healthy and diseased organs, and specifically applied to the analysis of intra-cell type variability in intestinal organoids.[132] While conventional k-means clustering yielded a transcriptionally-distinct cluster of secretory cells, the RaceID evolution further sub-divided this population into goblet cells, Paneth cells, and enteroendocrine cells, confirming the existence of these rarer cells within the organoid epithelium.

5.3 Measuring melanoma cell heterogeneity using RNA FISH and GiniClust

While scRNA-seq is powerful in its comprehensive analysis capability, unavoidable random loss of transcripts from single cells make analysis of extremely rare cells challenging.[82] An alternative method that is less comprehensive in coverage of types of RNA analyzed, but is more sensitive, is RNA fluorescent *in situ* hybridization (FISH). RNA FISH enables the spatial visualization of RNA molecules within the fixed cell using image processing and quantification methods.[133–135] Individual RNA molecules can be counted within the cell when bound with a fluorescent probe and multiple iterations of hybridization, de-hybridization, and re-hybridization with new probes is possible to analyze nearly 20 different RNAs in the same fixed sample.[82]

While RaceID worked well in the intestine organoid cell heterogeneity analysis, in test analyses of 280 gene transcripts quantified by quantitative polymerase chain reaction (qPCR), the method was shown to be computationally taxing and over-segregated cell types, creating misleading cell clusters from only single cells. To overcome these problems, Jiang and colleagues developed GiniClust.[136] This method determines a Gini index for each RNA analyzed, where an index of zero means the cell population expresses it homogenously while a score of one means all transcripts are expressed by a single cell. Thus, housekeeping genes trend towards lower Gini indices while more variably transcribed genes have higher Gini indices. By performing clustering using transcripts above a threshold Gini index, the amount of data used for clustering is significantly reduced, accelerating computational analysis. Also, by requiring rare cell clusters to contain more than one cell this method limits over-segregation faults identified with RaceID, while also discretizing lineages more accurately.

RNA FISH and Gini indices were used by Shaffer and colleagues to identify rare cells within a 2D-cultured melanoma population.[82] Nineteen mRNAs were probed from the same sample with multiple iterations of hybridization and imaging. From this data set, cells expressing RNA with high Gini indices were identified. RNAs associated with chemotherapy resistance like NGRF, Serpine and AXL received high Gini indices, indicating significant transcriptional rarity.

6. Work-arounds

Because miniaturization and physiological relevance through accurate cell heterogeneity representation do not often lead to similar design requirements, we propose some short-term solutions to achieve physiologically-relevant cellular heterogeneity, at both the cell type and sub-cell type levels, including the representation of rare cell events. This is not a cure-all list, but focuses on simple microfluidic device-oriented strategies as starting points for the organ-on-a-chip developer considering the study of cell-to-cell variability.

6.1 Avoid over-miniaturization

Organ-on-a-chip devices are regarded for replicating a single organ microenvironment *in vitro*, but to ensure the inclusion of rare cells this may not make the most sense. Organs can be thought of as containing many adjacent microenvironments. By miniaturizing an organ by too great a scale, rare cells would likely be lost (**Figure 5A**). By increasing the cell number or "organ size" of the culture model, better heterogeneity may already be achieved through natural fluctuation of culture condition from cell to cell and microenvironment to microenvironment in the same culture system. This may be particularly important when trying to take advantage of naturally-occurring cellular heterogeneity such as may exist in biopsy-derived cell populations and low passage primary cells.

6.2 Increase replicates

If increasing organ compartment size to increase the likelihood of rare cell existence is not feasible within other design constraints, increasing the number of culture system replicates studied in parallel may have use, especially in comparing data from individual devices instead of merely taking averages (**Figure 5B**). What might be considered experimental noise or error could instead be attributed to actual differences from

one device to another. By looking at multiple separate device experiments together, variability in characteristics like cell metabolism and secretion levels may resemble what is encountered *in vivo* within a single organ.

6.3 Increase experiment duration to capture rare events

If rare cell phenotypes arise over time in a defined or stochastic manner, on-chip models with a desired number of cells may require a longer period of study to capture those events. As evidenced in the report by Shaffer and colleagues, non-genetic transcriptional variability can be transient.[82] By increasing duration of the study and/or increasing time point density, brief or phenotypic events may be captured that are useful for validating the characteristics of an organ-on-a-chip (**Figure 5C**).

6.4 Organically engineer the microenvironment to create heterogeneity

We focus on a few examples from the intestine-on-a-chip field to highlight two major strategies for engineering devices with increased cellular heterogeneity. One strategy is to engineer biomaterials to promote cellular heterogeneity within an organ-on-a-chip. This was recently displayed by Wang, Allbritton and colleagues in multiple reports showing the potential to use collagen-based biomaterials to maintain the culture heterogeneity and segregation of tissue stem cells and differentiated cells that resembles the colonic and intestinal crypt and villi.[137,138] Two architectures were validated for maintaining cell heterogeneity from the harvested colonic crypts a flat substrate and on a micro-fabricated substrate with molded crypt and villi.

The other strategy is to first create physiological cell heterogeneity in organoids, then transfer those cells into organ-on-a-chip devices. To enable the study of a heterogeneous population of cells on-chip, early reports have been published on dissociating cultured organoids and seeding their cell constituents in microfluidic channels for controlled study (**Figure 5D**).[127,139] In the account by Kasendra and colleagues, this yielded an on-chip cultures with multiple differentiated epithelial cell types including an overall transcriptional signature that more closely resembled sampled intestinal tissue from where the primary cells used in the experiment were derived.

Organoids have at least two advantages over conventional 2D culture or organ-on-a-chip cultures: the ability to provide a more physiological 3D microenvironment and the ability to mature and be maintained

over many months, longer than is currently possible with organ-on-a-chip systems. In this strategy, instead of seeding cells in an organ-on-a-chip device to study, multiple research groups have successfully recapitulated tissue heterogeneity through growing 3D human organoid cultures. Human intestinal organoids were shown by Spence and colleagues to form *in vivo*-like structures from induced pluripotent stem cells (iPSCs) with crypt-like appendages branching off the organoid, where tissue stem cells remained localized with more differentiated cells migrating away.[124] Furthermore, Miller, Spence and colleagues adapted their strategy for applications in studying fetal lung development, by inducing iPSCs to select a lineage reminiscent of lung bud progenitor cells, observing similar heterogeneous cell patterning to *in vivo* development.[101] Differentiation of stem cells in an organoid enables longer term culture and reliable maturation to create a desired cell population for shorter-term microchannel experiments. While organoids can provide a diverse population of cells that arise from a stem cell, tissues and tumors have a diversity of stem cells as well. To maintain the stem cell diversity of the original tissue as much as possible, it is important to sample tissues from multiple different locations, culture all cells from a biopsy or tissue sample without pre-sorting the cells, and study as many organoids as possible.[140]

7. Conclusions

This review considers the emerging field of studying cell heterogeneity as an area of opportunity for developing organ-on-a-chip technologies that are more physiological and predictive of human health and disease. In addition to well-acknowledged cellular differences between species, demographics, organs, and cell types, the latest single cell analysis methods are discerning new sub-types within cell types originally considered to be a single type, such as a cancer cell or epithelial cell. Furthermore, there are increasingly prominent roles for rare cells that arise from temporary and transient cell-to-cell variabilities, as shown in examples where drug treatment can give rise to a population of distinct and stable persister cells resistant to cancer therapy from a rare population of cells initially only expressing high levels of drug resistance genes.[82]

The latest analysis and insights into cell heterogeneity can provide some mechanistic insights and quantifiable parameters to understand outlier results as well as to provide estimates on the capabilities and limits of organ-on-a-chip technologies constructed from different cells, biomaterials and culture conditions.

For example, on-chip cell heterogeneity and plasticity may be compared with *in vivo* tissues to identify organs or diseases for which the on-chip and *in vivo* cell types, cell heterogeneity, and cell plasticity match better. Experimenting with cells in organ-on-a-chip systems that are sourced from organoids known to have the required physiologically-relevant level of cell heterogeneity is a strategy of increasing promise in this regard. Analysis of cell heterogeneity can even be a measure of cell and organism aging as demonstrated recently in a comparisons of immune cells from young and old mice and humans.[31,32] Analysis of cell heterogeneity may also provide a benchmark for balancing the conflicting need to pursue higher throughput and miniaturization with the need to maintain physiological relevance.

While many of the required tools and biological expertise are already available, the next steps require more collaboration between organ-on-a-chip developers, single cell analysis experts, biomaterials scientists, bioinformaticians and cell biologists. In parallel with further consideration of organ-to-organ scaling,[8,16,17] media optimization, and metabolic control,[141] validation of organ-on-a-chip cell heterogeneity may be poised to bring improvement to overall generalizable potential and predictive accuracy.

8. Author contributions

DRM, TA and ST conceived the manuscript. DRM, TA and ST wrote the draft and reviewed and edited the manuscript.

9. Conflicts of interest and acknowledgements

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10. References

1 M. Ramirez, S. Rajaram, R. J. Steininger, D. Osipchuk, M. A. Roth, L. S. Morinishi, L. Evans, W. Ji, C. H. Hsu, K. Thurley, S. Wei, A. Zhou, P. R. Koduru, B. A. Posner, L. F. Wu and S. J. Altschuler, *Nat.*

Commun., , DOI:10.1038/ncomms10690.

2 Growing human Organs-on-a-Chip, https://www.roche.com/research_and_development/what_we_are_working_on/research_technologies/organs-on-a-chip-technology.htm, (accessed 3 April 2018).

3 A. K. White, M. VanInsberghe, O. I. Petriv, M. Hamidi, D. Sikorski, M. A. Marra, J. Piret, S. Aparicio and C. L. Hansen, *Proc. Natl. Acad. Sci.*, 2011, **108**, 13999–14004.

4 A. A. Pollen, T. J. Nowakowski, J. Shuga, X. Wang, A. A. Leyrat, J. H. Lui, N. Li, L. Szpankowski, B. Fowler, P. Chen, N. Ramalingam, G. Sun, M. Thu, M. Norris, R. Lebofsky, D. Toppani, D. W. Kemp, M. Wong, B. Clerkson, B. N. Jones, S. Wu, L. Knutsson, B. Alvarado, J. Wang, L. S. Weaver, A. P. May, R. C. Jones, M. A. Unger, A. R. Kriegstein and J. A. A. West, *Nat. Biotechnol.*, 2014, **32**, 1053–1058.

5 A. M. Klein, L. Mazutis, I. Akartuna, N. Tallapragada, A. Veres, V. Li, L. Peshkin, D. A. Weitz and M. W. Kirschner, *Cell*, 2015, **161**, 1187–1201.

6 T. H. Consortium, *The Human Cell Atlas White Paper*, 2017.

7 C. Y. McLean, P. L. Reno, A. A. Pollen, A. I. Bassan, T. D. Capellini, C. Guenther, V. B. Indjeian, X. Lim, D. B. Menke, B. T. Schaar, A. M. Wenger, G. Bejerano and D. M. Kingsley, *Nature*, 2011, **471**, 216–219.

8 C. Moraes, J. M. Labuz, B. M. Leung, M. Inoue, T.-H. Chun and S. Takayama, *Integr. Biol.*, 2013, **5**, 1149–1161.

9 K. Ronaldson-Bouchard and G. Vunjak-Novakovic, *Cell Stem Cell*, 2018, 22, 310–324.

10 G. Kumar, H. Lau and O. Laskin, *Cancer Chemother. Pharmacol.*, 2009, **63**, 1171–1175.

11 Immunopolymorphism Database Statistics, https://www.ebi.ac.uk/ipd/imgt/hla/stats.html, (accessed 4 April 2018).

12 N. Sachs, J. de Ligt, O. Kopper, E. Gogola, G. Bounova, F. Weeber, A. V. Balgobind, K. Wind, A. Gracanin, H. Begthel, J. Korving, R. van Boxtel, A. A. Duarte, D. Lelieveld, A. van Hoeck, R. F. Ernst, F. Blokzijl, I. J. Nijman, M. Hoogstraat, M. van de Ven, D. A. Egan, V. Zinzalla, J. Moll, S. F.

Boj, E. E. Voest, L. Wessels, P. J. van Diest, S. Rottenberg, R. G. J. Vries, E. Cuppen and H. Clevers, *Cell*, 2018, **172**, 373–386.e10.

13 M. Fujii, M. Shimokawa, S. Date, A. Takano, M. Matano, K. Nanki, Y. Ohta, K. Toshimitsu, Y. Nakazato, K. Kawasaki, T. Uraoka, T. Watanabe, T. Kanai and T. Sato, *Cell Stem Cell*, 2016, **18**, 827–838.

14 Rigor and Reproducibility, https://grants.nih.gov/reproducibility/index.htm, (accessed 16 April 2018).

15 Consideration of Sex as a Biological Variable in NIH□ funded Research, https://orwh.od.nih.gov/sites/orwh/files/docs/NOT-OD-15-102_Guidance.pdf, (accessed 13 April 2018).

16 J. P. Wikswo, E. L. Curtis, Z. E. Eagleton, B. C. Evans, A. Kole, L. H. Hofmeister and W. J. Matloff, *Lab Chip*, 2013, **13**, 3496.

17 M. B. Esch, A. S. T. Smith, J. M. Prot, C. Oleaga, J. J. Hickman and M. L. Shuler, *Adv. Drug Deliv. Rev.*, 2014, 69–70, 158–169.

18 I. Maschmeyer, A. K. Lorenz, K. Schimek, T. Hasenberg, A. P. Ramme, J. Hübner, M. Lindner, C. Drewell, S. Bauer, A. Thomas, N. S. Sambo, F. Sonntag, R. Lauster and U. Marx, *Lab Chip*, 2015, **15**, 2688–2699.

19 H. E. Abaci and M. L. Shuler, *Integr. Biol.*, 2015, **7**, 383–391.

20 D. Huh, H. Fujioka, Y.-C. Tung, N. Futai, R. Paine, J. B. Grotberg and S. Takayama, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 18886–18891.

21 D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin and D. E. Ingber, *Science*, 2010, **328**, 1662–8.

22 N. J. Douville, P. Zamankhan, Y.-C. Tung, R. Li, B. L. Vaughan, C.-F. Tai, J. White, P. J. Christensen, J. B. Grotberg and S. Takayama, *Lab Chip*, 2011, **11**, 609–619.

23 D. Huh, D. C. Leslie, B. D. Matthews, J. P. Fraser, S. Jurek, G. A. Hamilton, K. S. Thorneloe, M. A. McAlexander and D. E. Ingber, *Sci. Transl. Med.*, 2012, **4**, 159ra148.

24 J. D. Crapo, S. L. Young, E. K. Fram, K. E. Pinkerton, B. E. Barry and R. O. Crapo, *Am. Rev. Respir. Dis.*, 1983, **128**, S42-6.

25 T. J. Franks, T. V Colby, W. D. Travis, R. M. Tuder, H. Y. Reynolds, A. R. Brody, W. V Cardoso, R. G. Crystal, C. J. Drake, J. Engelhardt, M. Frid, E. Herzog, R. Mason, S. H. Phan, S. H. Randell, M. C. Rose, T. Stevens, J. Serge, M. E. Sunday, J. A. Voynow, B. M. Weinstein, J. Whitsett and M. C. Williams, *Proc. Am. Thorac. Soc.*, 2008, **5**, 763–766.

26 A. Sottoriva, H. Kang, Z. Ma, T. A. Graham, M. P. Salomon, J. Zhao, P. Marjoram, K. Siegmund, M. F. Press, D. Shibata and C. Curtis, *Nat. Genet.*, 2015, **47**, 209–216.

27 M. F. Lyon, *Nature*, 1961, **190**, 372–373.

28 S. L. Spencer, S. Gaudet, J. G. Albeck, J. M. Burke and P. K. Sorger, *Nature*, 2009, **459**, 428–432.

29 H. H. Chang, M. Hemberg, M. Barahona, D. E. Ingber and S. Huang, *Nature*, 2008, **453**, 544–547.

30 A. Nguyen, M. Yoshida, H. Goodarzi and S. F. Tavazoie, *Nat. Commun.*, , DOI:10.1038/ncomms11246.

31 C. P. Martinez-Jimenez, N. Eling, H. C. Chen, C. A. Vallejos, A. A. Kolodziejczyk, F. Connor, L. Stojic, T. F. Rayner, M. J. T. Stubbington, S. A. Teichmann, M. De La Roche, J. C. Marioni and D. T. Odom, *Science*, 2017, **355**, 1433–1436.

32 P. Cheung, F. Vallania, H. C. Warsinske, M. Donato, S. Schaffert, S. E. Chang, M. Dvorak, C. L. Dekker, M. M. Davis, P. J. Utz, P. Khatri and A. J. Kuo, *Cell*, 2018, **173**, 1385–1397.

33 E. L. Schneider and Y. Mitsui, *Proc. Natl. Acad. Sci. U. S. A.*, 1976, **73**, 3584–3588.

34 L. Hayflick, *Exp. Cell Res.*, 1965, **37**, 614–636.

35 ATCC, *Passage number effects in cell lines*, Manassas, 2016.

36 L. O'Driscoll, P. Gammell, E. McKiernan, E. Ryan, P. B. Jeppesen, S. Rani and M. Clynes, *J. Endocrinol.*, 2006, **191**, 665–676.

37 H. K. Lin, Y. C. Hu, L. Yang, S. Altuwaijri, Y. T. Chen, H. Y. Kang and C. Chang, *J. Biol. Chem.*, 2003, **278**, 50902–50907.

38 M. K. Oh, D. R. Scoles, C. Haipek, A. D. Strand, D. H. Gutmann, J. M. Olson and S. M. Pulst, *J. Cell.*

Biochem., 2003, **90**, 1068–1078.

39 M. Kozminsky, Y. Wang and S. Nagrath, *Curr. Opin. Chem. Eng.*, 2016, **11**, 59–66.

40 J. F. Swennenhuis, A. G. J. Tibbe, R. Levink, R. C. J. Sipkema and L. W. M. M. Terstappen, *Cytom. Part A*, 2009, **75**, 520–527.

41 G. Attard and J. S. de Bono, *Curr. Opin. Genet. Dev.*, 2011, 21, 50–58.

42 C. E. Meacham and S. J. Morrison, *Nature*, 2013, **501**, 328–337.

43 A. Marusyk and K. Polyak, *Biochim. Biophys. Acta - Rev. Cancer*, 2010, 1805, 105–117.

44 R. Portillo-Lara and N. Annabi, *Lab Chip*, 2016, **16**, 4063–4081.

45 Y. Choi, E. Hyun, J. Seo, C. Blundell, H. C. Kim, E. Lee, S. H. Lee, A. Moon, W. K. Moon and D. Huh, *Lab Chip*, 2015, **15**, 3350–3357.

46 T. Yu, Z. Guo, H. Fan, J. Song, Y. Liu, Z. Gao and Q. Wang, *Oncotarget*, 2016, **7**, 25593–25603.

47 X.-Y. Wang, Y. Pei, M. Xie, Z.-H. Jin, Y.-S. Xiao, Y. Wang, L.-N. Zhang, Y. Li and W.-H. Huang, *Lab Chip*, 2015, **15**, 1178–1187.

48 J. S. Jeon, S. Bersini, M. Gilardi, G. Dubini, J. L. Charest, M. Moretti and R. D. Kamm, *Proc. Natl. Acad. Sci.*, 2015, **112**, 214–219.

49 M. S. Nikshoar, M. A. Khayamian, S. Ansaryan, H. Sanati, M. Gharooni, L. Farahmand, F. Rezakhanloo, K. Majidzadeh-A, P. Hoseinpour, S. Dadgari, L. Kiani-M, M. Saqafi, M. Gity and M. Abdolahad, *Nat. Commun.*, , DOI:10.1038/s41467-017-02184-x.

50 H. Lee, W. Park, H. Ryu and N. L. Jeon, *Biomicrofluidics*, 2014, **8**, 054102.

51 A. B. Theberge, J. Yu, E. W. K. Young, W. A. Ricke, W. Bushman and D. J. Beebe, *Anal. Chem.*, 2015, **87**, 3239–3246.

52 M. Al-Hajj, M. S. Wicha, A. Benito-Hernandez, S. J. Morrison and M. F. Clarke, *Proc. Natl. Acad. Sci.*, 2003, **100**, 3983–3988.

53 C. Ginestier, M. H. Hur, E. Charafe-Jauffret, F. Monville, J. Dutcher, M. Brown, J. Jacquemier, P. Viens, C. G. Kleer, S. Liu, A. Schott, D. Hayes, D. Birnbaum, M. S. Wicha and G. Dontu, *Cell Stem Cell*, 2007, **1**, 555–567.

54 P. B. Gupta, C. M. Fillmore, G. Jiang, S. D. Shapira, K. Tao, C. Kuperwasser and E. S. Lander, *Cell*, 2011, **146**, 633–644.

55 M. Gerlinger, A. J. Rowan, S. Horswell, J. Larkin, D. Endesfelder, E. Gronroos, P. Martinez, N. Matthews, A. Stewart, P. Tarpey, I. Varela, B. Phillimore, S. Begum, N. Q. McDonald, A. Butler, D. Jones, K. Raine, C. Latimer, C. R. Santos, M. Nohadani, A. C. Eklund, B. Spencer-Dene, G. Clark, L. Pickering, G. Stamp, M. Gore, Z. Szallasi, J. Downward, P. A. Futreal and C. Swanton, *N. Engl. J. Med.*, 2012, **366**, 883–892.

56 S. P. Shah, A. Roth, R. Goya, A. Oloumi, G. Ha, Y. Zhao, G. Turashvili, J. Ding, K. Tse, G. Haffari, A. Bashashati, L. M. Prentice, J. Khattra, A. Burleigh, D. Yap, V. Bernard, A. McPherson, K. Shumansky, A. Crisan, R. Giuliany, A. Heravi-Moussavi, J. Rosner, D. Lai, I. Birol, R. Varhol, A. Tam, N. Dhalla, T. Zeng, K. Ma, S. K. Chan, M. Griffith, A. Moradian, S.-W. W. G. Cheng, G. B. Morin, P. Watson, K. Gelmon, S. Chia, S. F. Chin, C. Curtis, O. M. Rueda, P. D. Pharoah, S. Damaraju, J. MacKey, K. Hoon, T. Harkins, V. Tadigotla, M. Sigaroudinia, P. Gascard, T. Tlsty, J. F. Costello, I. M. Meyer, C. J. Eaves, W. W. Wasserman, S. Jones, D. Huntsman, M. Hirst, C. Caldas, M. A. Marra and S. Aparicio, *Nature*, 2012, **486**, 395–399.

57 E. R. F. and B. Volgestein, *Cell*, 1990, **61**, 759–767.

58 R. L. Siegel, K. D. Miller and A. Jemal, *CA. Cancer J. Clin.*, 2018, **68**, 7–30.

59 C. A. O'Brien, A. Pollett, S. Gallinger and J. E. Dick, *Nature*, 2007, **445**, 106–110.

60 L. Ricci-Vitiani, D. G. Lombardi, E. Pilozzi, M. Biffoni, M. Todaro, C. Peschle and R. De Maria, *Nature*, 2007, **445**, 111–115.

61 P. Dalerba, S. J. S. J. Dylla, I.-K. I. K. Park, R. Liu, X. Wang, R. W. Cho, T. Hoey, A. Gurney, E. H. Huang, D. M. Simeone, A. A. Shelton, G. Parmiani, C. Castelli and M. F. Clarke, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 10158–10163.

62 L. Vermeulen, M. Todaro, F. de Sousa Mello, M. R. Sprick, K. Kemper, M. Perez Alea, D. J. Richel, G. Stassi and J. P. Medema, *Proc. Natl. Acad. Sci.*, 2008, **105**, 13427–13432.

63 P. Dalerba, T. Kalisky, D. Sahoo, P. S. Rajendran, M. E. Rothenberg, A. A. Leyrat, S. Sim, J.

Okamoto, D. M. Johnston, D. Qian, M. Zabala, J. Bueno, N. F. Neff, J. Wang, A. A. Shelton, B. Visser, S. Hisamori, Y. Shimono, M. Van De Wetering, H. Clevers, M. F. Clarke and S. R. Quake, *Nat. Biotechnol.*, 2011, **29**, 1120–1127.

64 N. Barker, J. H. Van Es, J. Kuipers, P. Kujala, M. Van Den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P. J. Peters and H. Clevers, *Nature*, 2007, **449**, 1003–1007.

65 A. Merlos-Suárez, F. M. Barriga, P. Jung, M. Iglesias, M. V. Céspedes, D. Rossell, M. Sevillano, X. Hernando-Momblona, V. Da Silva-Diz, P. Muñoz, H. Clevers, E. Sancho, R. Mangues and E. Batlle, *Cell Stem Cell*, 2011, **8**, 511–524.

66 F. De Sousa E Melo, A. V Kurtova, J. M. Harnoss, N. Kljavin, J. D. Hoeck, J. Hung, J. E. Anderson, E. E. Storm, Z. Modrusan, H. Koeppen, G. J. P. Dijkgraaf, R. Piskol and F. J. De Sauvage, *Nature*, 2017, **543**, 676–680.

67 M. Shimokawa, Y. Ohta, S. Nishikori, M. Matano, A. Takano, M. Fujii, S. Date, S. Sugimoto, T. Kanai and T. Sato, *Nature*, 2017, **545**, 187–192.

68 C. Crosnier, D. Stamataki and J. Lewis, *Nat. Rev. Genet.*, 2006, 7, 349–359.

69 N. Barker, R. A. Ridgway, J. H. Van Es, M. Van De Wetering, H. Begthel, M. Van Den Born, E. Danenberg, A. R. Clarke, O. J. Sansom and H. Clevers, *Nature*, 2009, **457**, 608–611.

70 A. G. Schepers, H. J. Snippert, D. E. Stange, M. Van Den Born, J. H. Van Es, M. Van De Wetering and H. Clevers, *Science*, 2012, **337**, 730–735.

71 E. Sangiorgi and M. R. Capecchi, *Nat. Genet.*, 2008, **40**, 915–920.

72 L. Zhu, P. Gibson, D. S. Currle, Y. Tong, R. J. Richardson, I. T. Bayazitov, H. Poppleton, S. Zakharenko, D. W. Ellison and R. J. Gilbertson, *Nature*, 2009, **457**, 603–607.

73 H. Tian, B. Biehs, S. Warming, K. G. Leong, L. Rangell, O. D. Klein and F. J. De Sauvage, *Nature*, 2011, **478**, 255–259.

74 Y. Nakanishi, H. Seno, A. Fukuoka, T. Ueo, Y. Yamaga, T. Maruno, N. Nakanishi, K. Kanda, H. Komekado, M. Kawada, A. Isomura, K. Kawada, Y. Sakai, M. Yanagita, R. Kageyama, Y. Kawaguchi, M. M. Taketo, S. Yonehara and T. Chiba, *Nat. Genet.*, 2013, **45**, 98–103.

75 L. Chin, L. A. Garraway and D. E. Fisher, *Genes Dev.*, 2006, 20, 2149–2182.

76 M. J. C. Hendrix, E. A. Seftor, A. R. Hess and R. E. B. Seftor, *Oncogene*, 2003, **22**, 3070–3075.

77 D. Fang, T. K. Nguyen, K. Leishear, R. Finko, A. N. Kulp, S. Hotz, P. A. Van Belle, X. Xu, D. E. Elder and M. Herlyn, *Cancer Res.*, 2005, **65**, 9328–9337.

78 T. Schatton, G. F. Murphy, N. Y. Frank, K. Yamaura, A. M. Waaga-Gasser, M. Gasser, Q. Zhan, S. Jordan, L. M. Duncan, C. Weishaupt, R. C. Fuhlbrigge, T. S. Kupper, M. H. Sayegh and M. H. Frank, *Nature*, 2008, **451**, 345–349.

79 E. Quintana, M. Shackleton, M. S. Sabel, D. R. Fullen, T. M. Johnson and S. J. Morrison, *Nature*, 2008, **456**, 593–598.

80 R. Nazarian, H. Shi, Q. Wang, X. Kong, R. C. Koya, H. Lee, Z. Chen, M. K. Lee, N. Attar, H. Sazegar, T. Chodon, S. F. Nelson, G. McArthur, J. A. Sosman, A. Ribas and R. S. Lo, *Nature*, 2010, **468**, 973–977.

81 K. Trunzer, A. C. Pavlick, L. Schuchter, R. Gonzalez, G. A. McArthur, T. E. Hutson, S. J. Moschos, K. T. Flaherty, K. B. Kim, J. S. Weber, P. Hersey, G. V Long, D. Lawrence, P. A. Ott, R. K. Amaravadi, K. D. Lewis, I. Puzanov, R. S. Lo, A. Koehler, M. Kockx, O. Spleiss, A. Schell-Steven, H. N. Gilbert, L. Cockey, G. Bollag, R. J. Lee, A. K. Joe, J. A. Sosman and A. Ribas, *J. Clin. Oncol.*, 2013, **31**, 1767–1774.

82 S. M. Shaffer, M. C. Dunagin, S. R. Torborg, E. A. Torre, B. Emert, C. Krepler, M. Beqiri, K. Sproesser, P. A. Brafford, M. Xiao, E. Eggan, I. N. Anastopoulos, C. A. Vargas-Garcia, A. Singh, K. L. Nathanson, M. Herlyn and A. Raj, *Nature*, 2017, **546**, 431–435.

83 S. K. Singh, I. D. Clarke, M. Terasaki, V. E. Bonn, C. Hawkins, J. Squire and P. B. Dirks, *Cancer Res.*, 2003, **63**, 5821–8.

84 S. K. Singh, C. Hawkins, I. D. Clarke, J. A. Squire, J. Bayani, T. Hide, R. M. Henkelman, M. D. Cusimano and P. B. Dirks, *Nature*, 2004, **432**, 396–401.

85 D. Beier, P. Hau, M. Proescholdt, A. Lohmeier, J. Wischhusen, P. J. Oefner, L. Aigner, A. Brawanski, U. Bogdahn and C. P. Beier, *Cancer Res.*, 2007, **67**, 4010–4015.

86 A. Sottoriva, I. Spiteri, S. G. M. Piccirillo, A. Touloumis, V. P. Collins, J. C. Marioni, C. Curtis, C. Watts, S. Tavaré and S. Tavare, *Proc Natl Acad Sci U S A*, 2013, **110**, 4009–4014.

87 R. Chen, M. C. Nishimura, S. M. Bumbaca, S. Kharbanda, W. F. Forrest, I. M. Kasman, J. M. Greve, R. H. Soriano, L. L. Gilmour, C. S. Rivers, Z. Modrusan, S. Nacu, S. Guerrero, K. A. Edgar, J. J. Wallin, K. Lamszus, M. Westphal, S. Heim, C. D. James, S. R. VandenBerg, J. F. Costello, S. Moorefield, C. J. Cowdrey, M. Prados and H. S. Phillips, *Cancer Cell*, 2010, **17**, 362–75.

88 J. L. Wilding and W. F. Bodmer, *Cancer Res.*, 2014, 74, 2377–2384.

89 T. Kondo, T. Setoguchi and T. Taga, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 781–6.

90 L. Patrawala, T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool and D. G. Tang, *Cancer Res.*, 2005, **65**, 6207–6219.

91 P. P. Szotek, R. Pieretti-Vanmarcke, P. T. Masiakos, D. M. Dinulescu, D. Connolly, R. Foster, D. Dombkowski, F. Preffer, D. T. MacLaughlin and P. K. Donahoe, *Proc. Natl. Acad. Sci.*, 2006, **103**, 11154–11159.

92 Y. Zen, T. Fujii, S. Yoshikawa, H. Takamura, T. Tani, T. Ohta and Y. Nakanuma, *Am. J. Pathol.*, 2007, **170**, 1750–1762.

93 H. Li, X. Chen, T. Calhoun-Davis, K. Claypool and D. G. Tang, *Cancer Res.*, 2008, **68**, 1820–1825.

94 M. J. Pfeiffer and J. A. Schalken, *Eur. Urol.*, 2010, **57**, 246–255.

95 J. Chung, P. N. Ingram, T. Bersano-Begey and E. Yoon, *Biomicrofluidics*, 2014, **8**, 064103.

96 P. Marcato, C. A. Dean, P. Da, R. Araslanova, M. Gillis, M. Joshi, L. Helyer, L. Pan, A. Leidal, S. Gujar, C. A. Giacomantonio and P. W. K. Lee, *Stem Cells*, 2011, **29**, 32–45.

97 A. K. Croker, D. Goodale, J. Chu, C. Postenka, B. D. Hedley, D. A. Hess and A. L. Allan, *J. Cell. Mol. Med.*, 2009, **13**, 2236–2252.

98 E. Lefrançais, G. Ortiz-Muñoz, A. Caudrillier, B. Mallavia, F. Liu, D. M. Sayah, E. E. Thornton, M. B. Headley, T. David, S. R. Coughlin, M. F. Krummel, A. D. Leavitt, E. Passegué and M. R. Looney, *Nature*, 2017, **544**, 105–109.

99 K. H. Benam, R. Villenave, C. Lucchesi, A. Varone, C. Hubeau, H.-H. Lee, S. E. Alves, M. Salmon, T.

C. Ferrante, J. C. Weaver, A. Bahinski, G. A. Hamilton and D. E. Ingber, *Nat Meth*, 2016, **13**, 151–157.

100 B. Treutlein, D. G. Brownfield, A. R. Wu, N. F. Neff, G. L. Mantalas, F. H. Espinoza, T. J. Desai, M. A. Krasnow and S. R. Quake, *Nature*, 2014, **509**, 371–375.

101 A. J. Miller, D. R. Hill, M. S. Nagy, Y. Aoki, B. R. Dye, A. M. Chin, S. Huang, F. Zhu, E. S. White, V. Lama and J. R. Spence, *Stem Cell Reports*, 2017, 10, 101–119.

102 C. Basbaum, *Annu. Rev. Physiol.*, 1990, **52**, 97–113.

103 T. J. Mathews and M. F. MacDorman, *Natl. Vital Stat. Rep.*, 2007, **55**, 1–32.

104 M. Guilliams, I. De Kleer, S. Henri, S. Post, L. Vanhoutte, S. De Prijck, K. Deswarte, B. Malissen, H. Hammad and B. N. Lambrecht, *J. Exp. Med.*, 2013, **210**, 1977–1992.

105 D. Hashimoto, A. Chow, C. Noizat, P. Teo, M. B. Beasley, M. Leboeuf, C. D. Becker, P. See, J. Price, D. Lucas, M. Greter, A. Mortha, S. W. Boyer, E. C. Forsberg, M. Tanaka, N. van Rooijen, A. García-Sastre, E. R. Stanley, F. Ginhoux, P. S. Frenette and M. Merad, *Immunity*, 2013, **38**, 792–804.

106 T. Hussell and T. J. Bell, *Nat. Rev. Immunol.*, 2014, 14, 81–93.

107 R. Pabst and T. Tschernig, *Anat. Embryol. (Berl).*, 1995, 192, 293–299.

108 A. Ferguson, *Gut*, 1977, **18**, 921–937.

109 E. Goto, H. Kohrogi, N. Hirata, K. Tsumori, S. Hirosako, J. Hamamoto, K. Fujii, O. Kawano and M. Ando, *Am. J. Respir. Cell Mol. Biol.*, 2000, **22**, 405–411.

110 V. Groh, A. Steinle, S. Bauer and T. Spies, *Science*, 1998, **279**, 1737–1740.

111 M. Lahn, *J. Mol. Med.*, 2000, **78**, 409–425.

112 Y. Chvatchko, *J. Exp. Med.*, 1996, **184**, 2353–2360.

113 T. Tschernig and R. Pabst, *Pathobiology*, 2000, 68, 1–8.

114 J. E. Moyron-Quiroz, J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund and T. D. Randall, *Nat. Med.*, 2004, **10**, 927–934.

115 M. Yamada, H. Kubo, S. Kobayashi, K. Ishizawa, M. He, T. Suzuki, N. Fujino, H. Kunishima, M. Hatta, K. Nishimaki, T. Aoyagi, K. Tokuda, M. Kitagawa, H. Yano, H. Tamamura, N. Fujii and M.

Kaku, *Cell. Mol. Immunol.*, 2011, **8**, 305–314.

116 M. Fournier, F. Lebargy, F. L. R. Ladurie, E. Lenormand and R. Pariente, *Am. Rev. Respir. Dis.*, 1989, **140**, 737–742.

117 F. Radtke and H. Clevers, *Science*, 2005, 307, 1904–1909.

118 L. W. Peterson and D. Artis, *Nat. Rev. Immunol.*, 2014, 14, 141–153.

119 T. Sato, R. G. Vries, H. J. Snippert, M. Van De Wetering, N. Barker, D. E. Stange, J. H. Van Es, A. Abo, P. Kujala, P. J. Peters and H. Clevers, *Nature*, 2009, **459**, 262–265.

120 G. Noel, N. W. Baetz, J. F. Staab, M. Donowitz, O. Kovbasnjuk, M. F. Pasetti and N. C. Zachos, *Sci. Rep.*, , DOI:10.1038/srep45270.

121 K. A. Heel, R. D. McCauley, J. M. Papadimitriou and J. C. Hall, *J. Gastroenterol. Hepatol.*, 1997, 12, 122–136.

122 S. W. Craig and J. J. Cebra, *J. Exp. Med.*, 1971, **134**, 188–200.

123 P. H. Dedhia, N. Bertaux-Skeirik, Y. Zavros and J. R. Spence, *Gastroenterology*, 2016, 150, 1098–1112.

124 J. R. Spence, C. N. Mayhew, S. A. Rankin, M. F. Kuhar, J. E. Vallance, K. Tolle, E. E. Hoskins, V. V. Kalinichenko, S. I. Wells, A. M. Zorn, N. F. Shroyer and J. M. Wells, *Nature*, 2011, **470**, 105–110.

125 K. W. McCracken, J. C. Howell, J. M. Wells and J. R. Spence, *Nat. Protoc.*, 2011, **6**, 1920–1928.

126 C. L. Watson, M. M. Mahe, J. Múnera, J. C. Howell, N. Sundaram, H. M. Poling, J. I. Schweitzer, J. E. Vallance, C. N. Mayhew, Y. Sun, G. Grabowski, S. R. Finkbeiner, J. R. Spence, N. F. Shroyer, J. M. Wells and M. A. Helmrath, *Nat. Med.*, 2014, **20**, 1310–1314.

127 M. J. Workman, J. P. Gleeson, E. J. Troisi, H. Q. Estrada, S. J. Kerns, C. D. Hinojosa, G. A. Hamilton, S. R. Targan, C. N. Svendsen and R. J. Barrett, *Cell. Mol. Gastroenterol. Hepatol.*, , DOI:10.1016/j.jcmgh.2017.12.008.

128 S. C. Bendall, E. F. Simonds, P. Qiu, E. A. D. Amir, P. O. Krutzik, R. Finck, R. V Bruggner, R. Melamed, A. Trejo, O. I. Ornatsky, R. S. Balderas, S. K. Plevritis, K. Sachs, D. Pe'er, S. D. Tanner and G. P. Nolan, *Science*, 2011, **332**, 687–696.

129 M. Setty, M. D. Tadmor, S. Reich-Zeliger, O. Angel, T. M. Salame, P. Kathail, K. Choi, S. Bendall, N.

Friedman and D. Pe'Er, *Nat. Biotechnol.*, 2016, **34**, 637–645.

130 G. Quadrato, T. Nguyen, E. Z. Macosko, J. L. Sherwood, S. M. Yang, D. R. Berger, N. Maria, J. Scholvin, M. Goldman, J. P. Kinney, E. S. Boyden, J. W. Lichtman, Z. M. Williams, S. A. McCarroll and P. Arlotta, *Nature*, 2017, **545**, 48–53.

131 J. G. Camp, K. Sekine, T. Gerber, H. Loeffler-Wirth, H. Binder, M. Gac, S. Kanton, J. Kageyama, G. Damm, D. Seehofer, L. Belicova, M. Bickle, R. Barsacchi, R. Okuda, E. Yoshizawa, M. Kimura, H. Ayabe, H. Taniguchi, T. Takebe and B. Treutlein, *Nature*, 2017, **546**, 533–538.

132 D. Grün, A. Lyubimova, L. Kester, K. Wiebrands, O. Basak, N. Sasaki, H. Clevers and A. Van Oudenaarden, *Nature*, 2015, **525**, 251–255.

133 A. M. Femino, F. S. Fay, K. Fogarty and R. H. Singer, *Science*, 1998, **280**, 585–590.

134 A. Raj, P. van den Bogaard, S. A. Rifkin, A. van Oudenaarden and S. Tyagi, *Nat. Methods*, 2008, **5**, 877–879.

135 E. Lubeck and L. Cai, *Nat. Methods*, 2012, **9**, 743–748.

136 L. Jiang, H. Chen, L. Pinello and G. C. Yuan, *Genome Biol.*, , DOI:10.1186/s13059-016-1010-4.

137 Y. Wang, M. DiSalvo, D. B. Gunasekara, J. Dutton, A. Proctor, M. S. Lebhar, I. A. Williamson, J. Speer, R. L. Howard, N. M. Smiddy, S. J. Bultman, C. E. Sims, S. T. Magness and N. L. Allbritton, *CMGH*, 2017, **4**, 165–182.e7.

138 Y. Wang, D. B. Gunasekara, M. I. Reed, M. DiSalvo, S. J. Bultman, C. E. Sims, S. T. Magness and N. L. Allbritton, *Biomaterials*, 2017, **128**, 44–55.

139 M. Kasendra, A. Tovaglieri, A. Sontheimer-Phelps, S. Jalili-Firoozinezhad, A. Bein, A. Chalkiadaki, W. Scholl, C. Zhang, H. Rickner, C. A. Richmond, H. Li, D. T. Breault and D. E. Ingber, *Sci. Rep.*, , DOI:10.1038/s41598-018-21201-7.

140 S. F. Roerink, N. Sasaki, H. Lee-Six, M. D. Young, L. B. Alexandrov, S. Behjati, T. J. Mitchell, S. Grossmann, H. Lightfoot, D. A. Egan, A. Pronk, N. Smakman, J. Gorp, E. Anderson, S. J. Gamble, C. Alder, M. Wetering, P. J. Campbell, M. R. Stratton and H. Clevers, *Nature*, , DOI:10.1038/s41586-018-0024-3.

141 J. M. Labuz, C. Moraes, D. R. Mertz, B. M. Leung and S. Takayama, *TECHNOLOGY*, 2017, **05**, 42–59.

142 A. D. Boiko, O. V Razorenova, M. Van De Rijn, S. M. Swetter, D. L. Johnson, D. P. Ly, P. D. Butler, G. P. Yang, B. Joshua, M. J. Kaplan, M. T. Longaker and I. L. Weissman, *Nature*, 2010, **466**, 133–137.

143 G. Civenni, A. Walter, N. Kobert, D. Mihic-Probst, M. Zipser, B. Belloni, B. Seifert, H. Moch, R. Dummer, M. Van Den Broek and L. Sommer, *Cancer Res.*, 2011, **71**, 3098–3109.

144 A. Roesch, B. Becker, S. Meyer, P. Wild, C. Hafner, M. Landthaler and T. Vogt, *Mod. Pathol.*, 2005, **18**, 1249–1257.

145 A. Roesch, M. Fukunaga-Kalabis, E. C. Schmidt, S. E. Zabierowski, P. A. Brafford, A. Vultur, D. Basu, P. Gimotty, T. Vogt and M. Herlyn, *Cell*, 2010, **141**, 583–594.

146 I. Tirosh, B. Izar, S. M. Prakadan, M. H. Wadsworth, D. Treacy, J. J. Trombetta, A. Rotem, C. Rodman, C. Lian, G. Murphy, M. Fallahi-Sichani, K. Dutton-Regester, J. R. Lin, O. Cohen, P. Shah, D. Lu, A. S. Genshaft, T. K. Hughes, C. G. K. Ziegler, S. W. Kazer, A. Gaillard, K. E. Kolb, A. C. Villani, C. M. Johannessen, A. Y. Andreev, E. M. Van Allen, M. Bertagnolli, P. K. Sorger, R. J. Sullivan, K. T. Flaherty, D. T. Frederick, J. Jané-Valbuena, C. H. Yoon, O. Rozenblatt-Rosen, A. K. Shalek, A. Regev and L. A. Garraway, *Science*, 2016, **352**, 189–196.

147 T. Lapidot, C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M. A. Caligiuri and J. E. Dick, *Nature*, 1994, **367**, 645–648.

Table 1. Ratios for tumorigenic cells in various human cancers.			
Cancer	**Tumorigenic cell marker**	**Ratio/percentage**	**Reference**
Breast cancer	$CD44^{+}CD24^{-/low}$	1:20 – 1:40 (2.5-5%)	52
	$ESA^{+}CD44^{+}CD24^{-/low}$	~1:166 (0.6%)	52
	ALDH1	unknown	53
Colorectal cancer	CD133/prominin-1	$1{:}5.7 \times 10^{4}$	59,60
	$EpCAM^{+}CD44^{+}$	~1:18 (mean: 5.4%)	61
	$EpCAM^{+}CD166^{+}$	~1:16 (mean: 6%)	61
	CD29 CD24 Lgr5 β-catenin	unknown	62
	KRT20	unknown	63
Small intestinal cancer	Lgr5	1:10 – 1:20 (5-10%)	70
	Bmi1	unknown	71
	Dclk1	unknown	74
Melanoma	ABCB5	1:1 million 1:4	78 79
	CD271	1:6	142,143
	JARID1B	1:10 - 1:20 (5-10%)	144,145
	MITF	Dependent on selection method	146
Glioblastoma	CD133/prominin-1	~1:16 - 1:28 (3.5-6.1%)	83

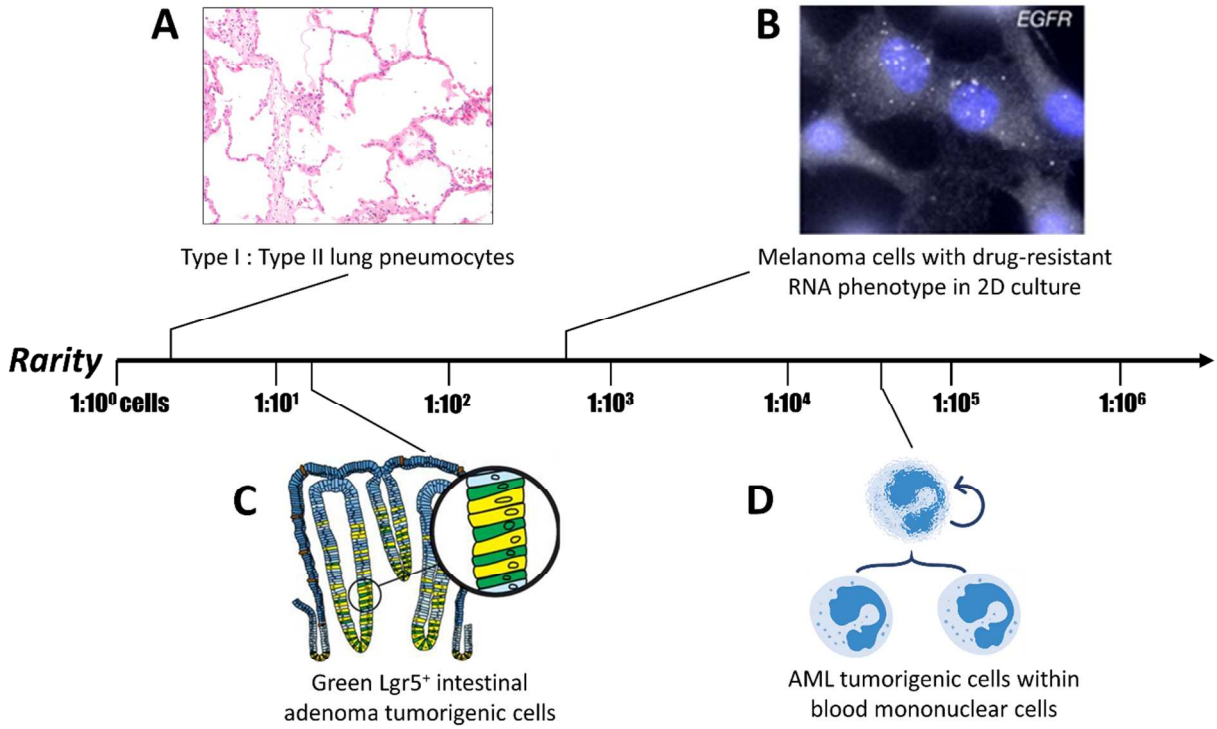

Figure 1: Cell heterogeneity may be considered on a range of levels in physiology and disease. (A) Lung alveolar epithelial cells, or pneumocytes, are classified in two subtypes, which occur, approximately, in a 1:2 ratio in the lung.[24] (B) Among populations of cancer cells, transcriptional heterogeneity may lead to rare drug-resistant cells as was shown by Shaffer and colleagues in a melanoma cell population.[82] (C,D) Additionally, tumorigenic cells, when identified by particular markers, can be found across the spectrum of rarity depending on the tumor site, but also the patient. $Lgr5^+$ intestinal adenoma cells[70] and $CD34^+CD38^-$ acute myeloid leukemia cells[147] are such examples.

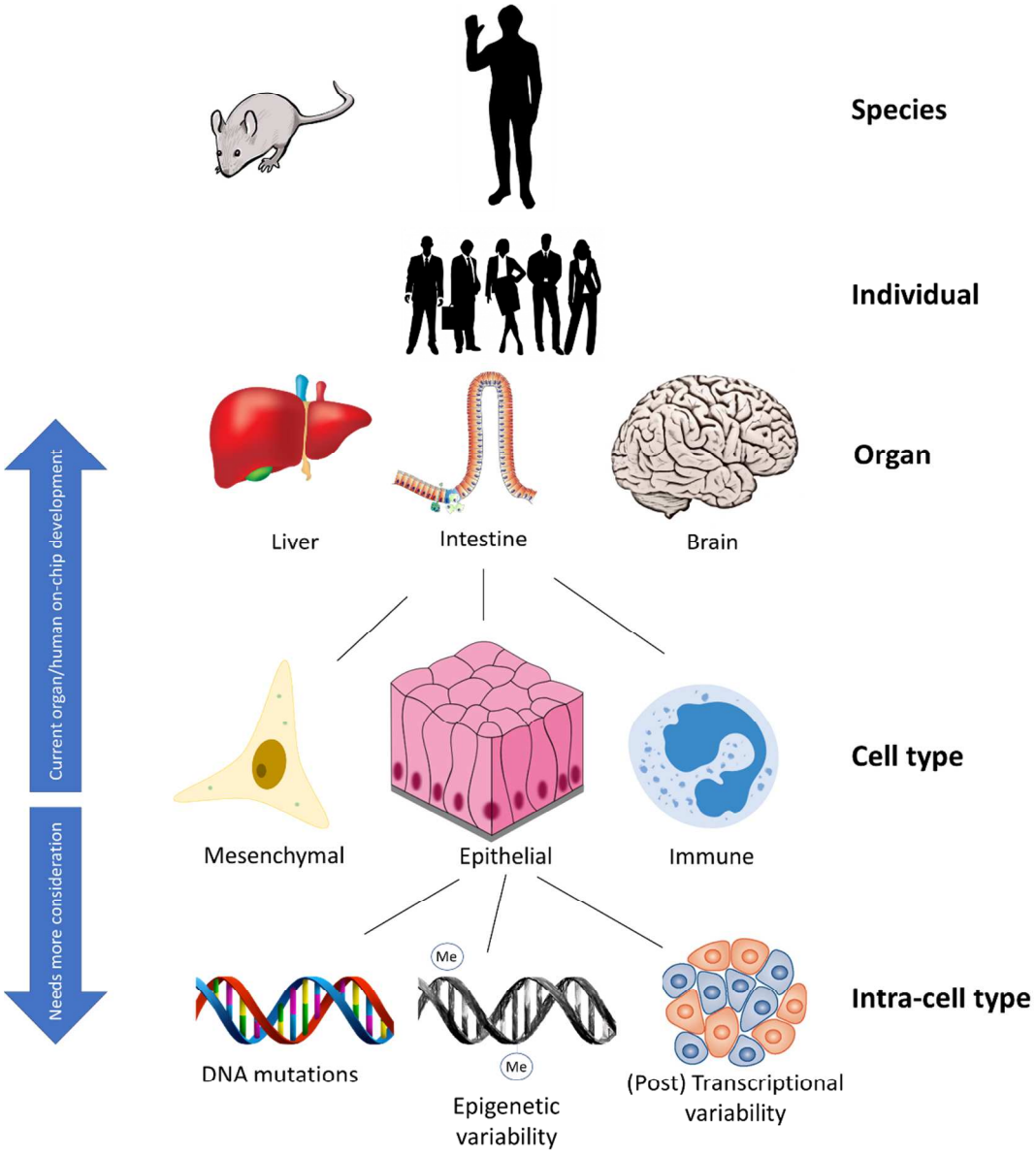

Figure 2: A hierarchy of cell-to-cell variability is illustrated. While much of organ-on-a-chip development has considered tissue heterogeneity (co-culturing multiple cell types within the same organ) and body/human-on-a-chip development has considered organ-to-organ differences and scaling, a deeper level of cell-to-cell variability exists that is less-characterized by organ-on-a-chip engineers. This heterogeneity within a single cell type may arise through genetic mutations, but also specific epigenetic mechanisms which are largely unknown. Phenotypic heterogeneity is characterizable, however, with advancements in the past few years using single cell techniques, and these techniques may have application in organ-on-a-chip development by assessing cells used in the culture model to the heterogeneity found in the microenvironment to be mimicked.

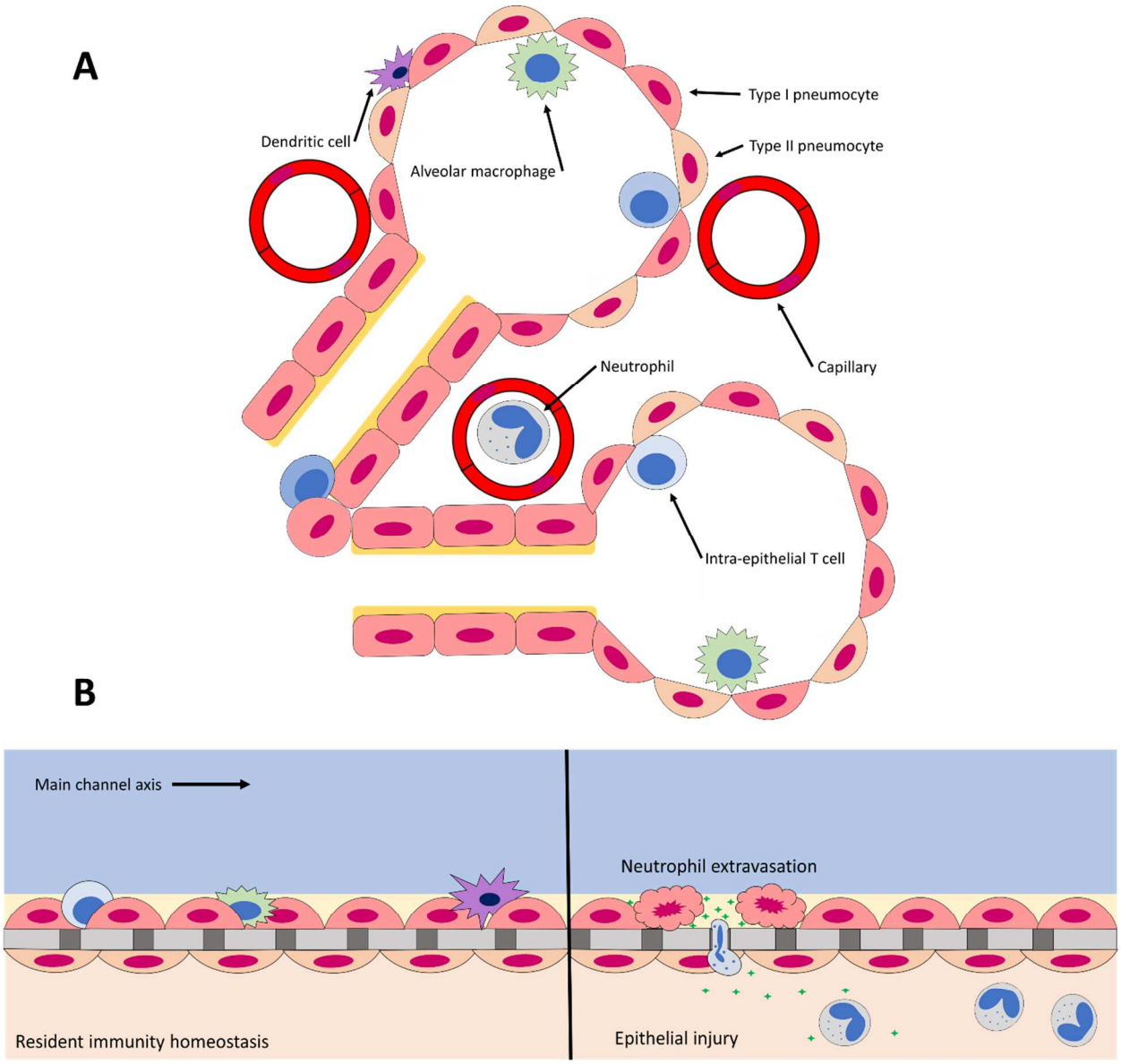

Figure 3: Modeling the immune component of the small airway microenvironment on-chip. (A) Multiple immune cell types: alveolar macrophages, intraepithelial T cells and dendritic cells reside within the epithelium to defend against pathogens with innate or adaptive immune responses. Neutrophils do not generally leave the circulation at homeostasis, but may extravasate to participate in an immune response. (B) Two lung-on-a-chip adaptations of the lung microenvironment are depicted. Left is a generalized, homeostatic lung with immune component consisting of resident immune cells like alveolar macrophages, intraepithelial T cells or dendritic cells. Right is a proposed on-chip model of dynamic immunity with airway epithelial injury leading to extravasation of chemotactic neutrophils at the wound site.

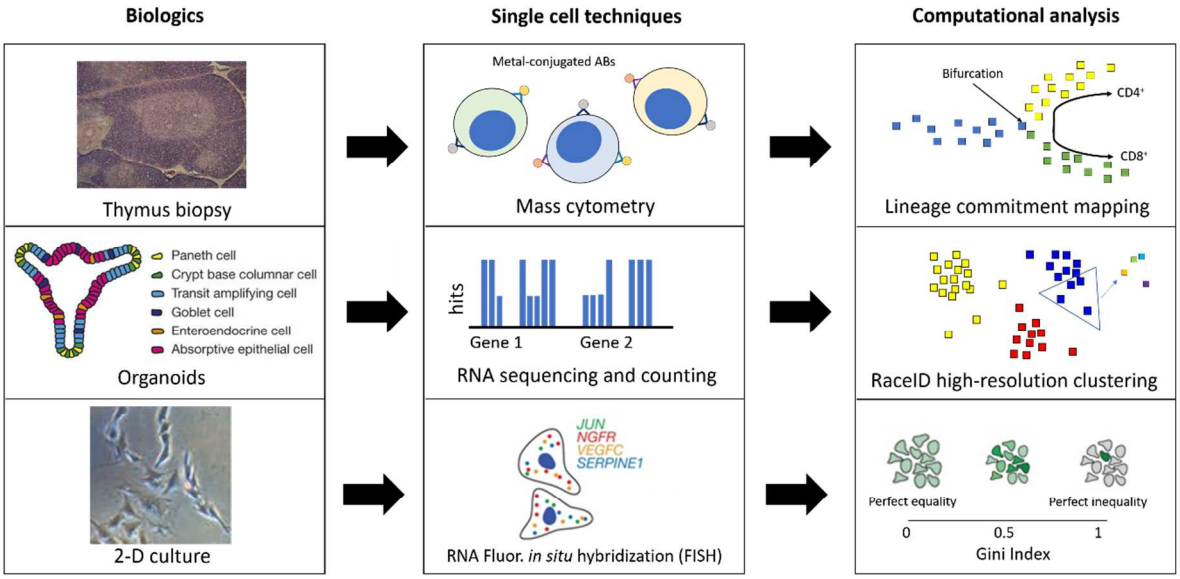

Figure 4: Cell-to-cell variability has been assessed in excised tissues, organoids and in 2D cultures. General workflow for cell-to-cell heterogeneity characterization are depicted using three example cases stacked top to bottom. Setty and colleagues collected proteomic data from primary thymocytes using mass cytometry and developed an algorithm to map cells along lineage progression.[129] Grün and colleagues evaluated cell heterogeneity in intestinal organoids using scRNA-seq as well, but proposed a more advanced clustering algorithm known as RaceID (rare cell identification) to resolve finer cell-to-cell differences, leaving cell type clusters with as few as a single cell.[132] Even a 2D-cultured cell line was shown by Shaffer and colleagues to contain RNA copy-number variability when using RNA FISH. Cell population heterogeneity was quantifiable using the Gini index, which for certain genes correlated with chemotherapy resistance.[82] Far fewer accounts have been published on cell-to-cell heterogeneity within organ-on-a-chip devices, and these approaches may provide key insights to advance their predictive accuracy.

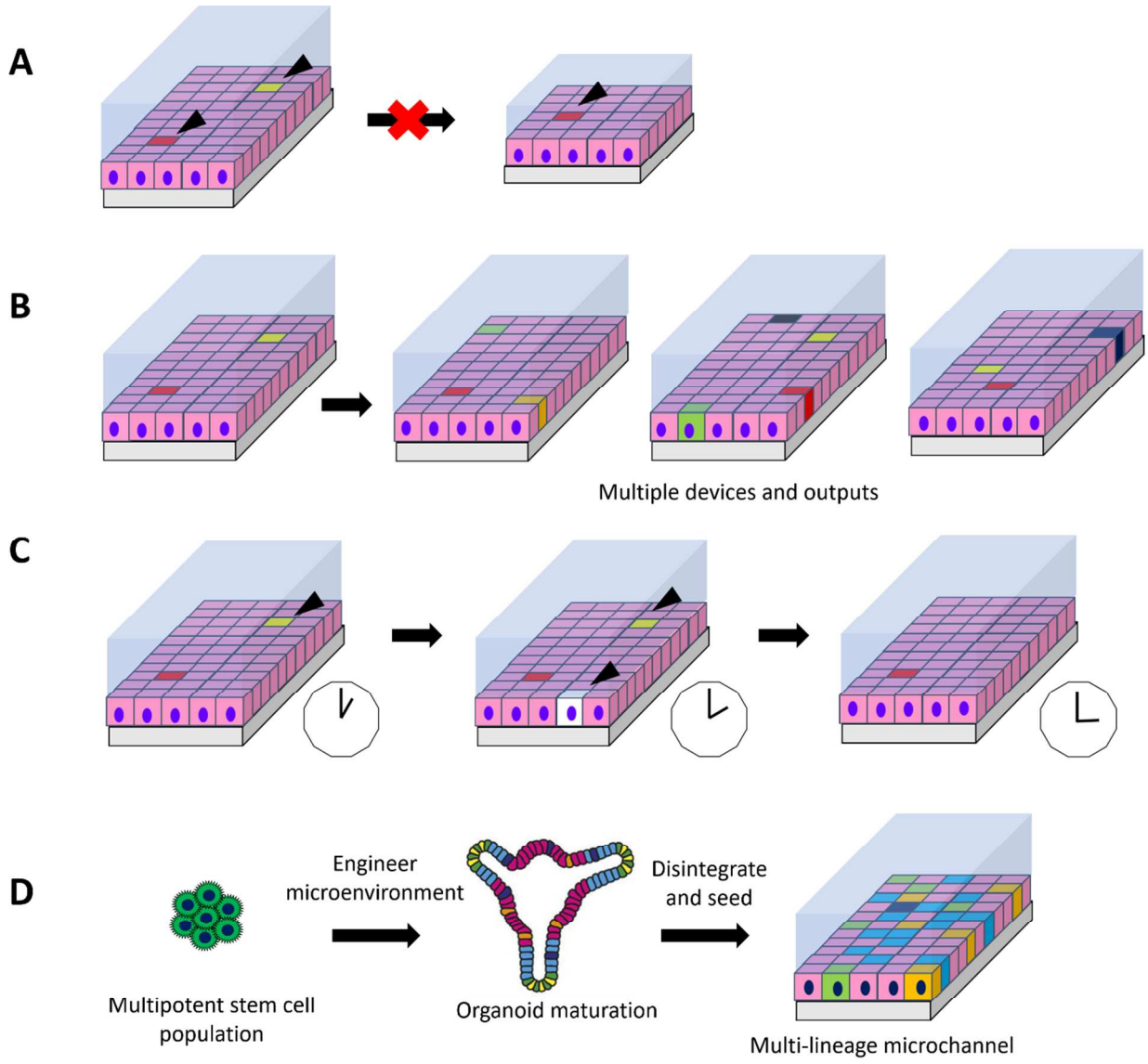

Figure 5: Visual schematics of workarounds for characterizing cell-to-cell variability and rare cells or events are depicted. (A) By miniaturizing too much or by using too few cells, rare phenotypes (indicated by black arrowheads) can be lost. (B) Increasing experimental replicates will create microenvironments with rare cells, providing a range of outputs. (C) Increasing experimental duration or frequency of data acquisition may capture outputs from transient rare phenotypes (indicated by black arrowheads). (D) Beginning an organ-on-a-chip experiment with multipotent stem cells, in combination with biochemical signals and matrix will create a heterogeneous cell population including progenitors and multiple lineages.

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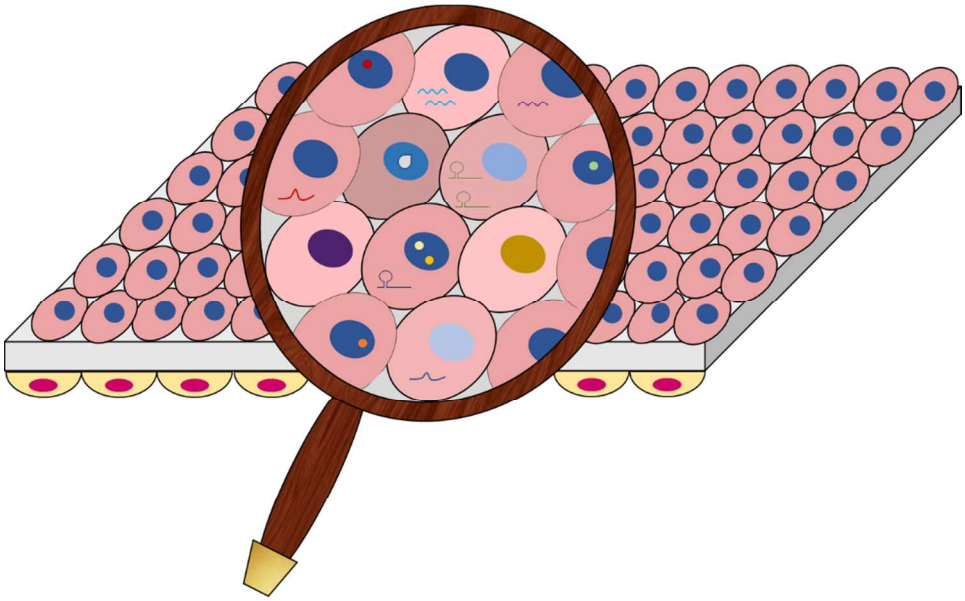

To improve predictive efficacy of organ-on-a-chip devices, developers must consider cell heterogeneity.