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# Mapping human cell phenotypes to genotypes with single-cell genomics

**Review Article** 

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| 1        | Mapping human cell phenotypes to genotypes with single-cell genomics                                   |  |  |
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| 9        |  |  |  |
| 10       | ABSTRACT   |  |  |
| 11       |  |  |  |
| 12       | The cumulative activity of all of our cells, with their myriad of interactions, life histories, and    |  |  |
| 13       | environmental experiences, gives rise to a condition that is uniquely numan, and specific to           |  |  |
| 15       | develop how they vary between individuals, and how they fail in disease. Single-cell genomics          |  |  |
| 16       | has revolutionized this endeavor as sequencing-based methods provide a means to                        |  |  |
| 17       | quantitatively annotate cell states based on high-information content and high-throughput              |  |  |
| 18       | measurements. Together with advances in stem cell biology and gene editing, we are in the              |  |  |
| 19       | midst of a fascinating journey to understand the cellular phenotypes that compose human                |  |  |
| 20       | bodies and how the human genome is used to build and maintain each cell. Here we will review           |  |  |
| 21       | recent advances into how single-cell genomics is being used to develop personalized                    |  |  |
| 22<br>23 | our cumulative cellular phenotypes   |  |  |
| 24       |  |  |  |
| 25       | Phenotyping in the single-cell sequencing era  |  |  |
| 26       |  |  |  |
| 27       | Phenotype can mean many things, but in general it is a way to classify a set of properties that        |  |  |
| 28       | arise from the interaction of an individual's genotype with its environment. It is a reductionist, yet |  |  |
| 29       | powerful, approach to take numan phenotyping down to the level of single cells and use                 |  |  |
| 31       | tissue/system level. Single-cell sequencing technologies can measure thousands of individual           |  |  |
| 32       | features per cell for thousands of cells at a time, providing a quantitative and ultra-high            |  |  |
| 33       | resolution snapshot of cell and molecular states composing a human tissue, organ, or other             |  |  |
| 34       | biosystem. Currently, there are protocols available to measure the RNA content, DNA sequence           |  |  |
| 35       | and methylation status, chromatin structure and accessibility, and protein composition in single       |  |  |
| 36       | cells(1). In addition to molecular features, cell histories can be measured in certain scenarios       |  |  |
| 31<br>20 | where mutations have arisen in nuclear(2-4) or mitochondrial(5) DNA that distinguish lineages.         |  |  |
| 39       | is possible to record cell fate histories and infer lineage trees using reporter barcodes and          |  |  |
| 40       | genetic scarring based on RNA-guided CRISPR (clustered regularly interspaced short                     |  |  |
| 41       | palindromic repeats)-associated (Cas) nucleases(6-9). So far, generally one or two single-cell         |  |  |
| 42       | measures have been used to phenotype individual cells within tissues in each experiment.               |  |  |
| 43       | However, the field is moving towards multimodal measurements from the same cell that capture           |  |  |
|          |  |  |  |

transcriptomic, genomic, epigenomic, and lineage states to enhance feature quantifications and
give a richer picture of a cell's phenotype at any given moment(*10-12*). These technologies are
foreign epigentiate to graphle with provide patience of "cell type", so cell properties converties.

46 forcing scientists to grapple with previous notions of "cell type", as cell properties can vary

discretely (type or subtype), as well as continuously (state), and classification may not always
follow rigid hierarchies(*13*). An additional layer of complexity arises in developing systems

49 where cell states and tissue morphologies are changing rapidly with time. Single-cell

- 50 sequencing captures transient states, and computational approaches enable trajectory(14) and
- 51 lineage(9) reconstructions.

52 The microenvironment of each cell can also be critical to a individual cell's phenotype. 53 Spatial transcriptomic and proteomic approaches based on multiplexed RNA hybridizations(15, 54 16) or protein immunohistochemistry(17), in situ sequencing(18-20), mass cytometry(21) or 55 other strategies(22) can be used to measure cell types and states in situ. In addition, methods 56 are being developed to analyze spatial locations of molecules within a cell and cells within a 57 tissue using barcoded oligonucleotides that can couple together when the molecules are close 58 enough to physically interact(23, 24). These pairwise interactions are encoded in DNA and can 59 be measured by high-throughput sequencing, and computational analyses enable spatial 60 reconstruction of cell interactions based on the molecular proximities.

61 Single-cell phenotyping has provided extraordinary atlases of model organisms that 62 span organ systems from the same animal(25-27), link cell morphologies with molecular 63 features(28), resolve cell type classifications(18, 29) and gradients of cell states (30, 31) 64 spatially, and assemble cell fates maps using lineage recording(7). This wave is extending to 65 developing, mature, and aged human organs(32, 33), identifying previously undocumented 66 human cell types(34-36) and providing a quantitative framework to classify human cell subtypes 67 and other states (37, 38). A major technical goal is to integrate (39, 40) all possible feature 68 detection methods (RNA, DNA, chromatin state, lineage, etc.) performed independently or in 69 combination in single cells, and render these measurements into three-dimensional spatial 70 volumes with subcellular and temporal resolution (Figure 1). By measuring cell heterogeneity 71 with such high information content techniques, emergent phenotypes at the tissue or biosystem 72 level (system phenotype) such as composition, regulatory states, cell interactions, spatial 73 constructions, and differentiation trajectories can be compared across environmental and 74 genetic conditions. Because human development is not deterministic, it will be interesting to see 75 how variable organ reconstructions are across different humans. Similar to the Human Genome 76 Project, the Human Cell Atlas (HCA) consortium will confront the challenge of how to generate a 77 reference organ map when the organ may look different in every person. The process of 78 assessing such variations using integrated multi-modal measurements, as is being proposed in 79 HCA projects over the next few years, will help to create common coordinated frameworks for 80 sampling tissues, processing samples and data, and computational comparisons between 81 methods and individuals.

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### 83 Disease associated cell phenotypes

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As each human and mouse organ is mapped at single-cell resolution across space and
time, a next phase of inquiry is to understand how genetic changes impact human phenotypes.
Disease association studies have used naturally occuring mutations to identify disease-causing

88 genes: these genes are Mendelian, have strong-effect coding mutations, or have been 89 experimentally validated. Disease-causing genes can be mapped to cell types by identifying the 90 cell population where the gene is expressed (multi-organ(41), kidney(42), cortex(43); Figure 2a). 91 A notable example was shown recently when researchers mapped out the cell types within the 92 adult human lung, and identified a new human cell type ("ionocyte"), which was the only type 93 that expressed CFTR at high levels and therefore likely mediates the lung pathology observed 94 in cystic fibrosis (35, 36). However, most disease associated mutations identified through 95 GWAS are likely regulatory, and the genes they regulate are currently unknown. Multimodel measures combining single-cell DNA. RNA, and chromatin can locate portions of the genome 96 97 that are active in a certain cell type or state providing a link from the regulatory genome to the 98 cell and even tissue phenotype. Future exploration into population measures of cell and tissue 99 phenotypes at the single-cell level(44, 45), similar to what has been done using bulk RNAseg of 100 adult human organs from the GTEx project(46), will be critical to identify the mechanism of 101 action of most GWAS variants.

102 In addition, researchers have started to phenotype human diseased tissues (Figure 2b), 103 thereby elucidating spatial glial neuron interactions in Amyotrophic Lateral Sclerosis(47), 104 identifying disease associated microglia in Alzheimers disease(48) and fibroblasts in Arthritis 105 (49), profiling pancreatic islets in health and type 2 diabetes (50), and identifying cellular rewiring 106 during colitis(51). These types of analyses promise to bring about a new phase of molecular 107 disease classification and diagnosis, and guide the development of therapies that can target the 108 specific cell types impacted by a given disease. This is already happening for various 109 cancers(52), however, there are major challenges requiring innovation to bring single-cell 110 sequencing technologies to patients with other disorders on a clinical scale. Protocols to 111 minimize cell loss during experiments could make it feasible to work with minute amounts of 112 input material (53). Apart from cancer, it is difficult to acquire tissue from most diseased 113 microenvironments in patients, and miniaturzied biopsies from healthy and diseased regions of 114 a tissue could open up disorders that lack clear molecular phenotypes. Industrialization of the 115 single-cell –omics pipeline from sample preparation and sequencing, to data analysis is also 116 required. This could include protocol optimizations to reduce cost of cell throughput while 117 retaining sensitivity (25, 54, 55), to increase sample multiplexing based on reference 118 polymorphisms (45) or tagging (56, 57), and to compress phenotyping by experimentally 119 enriching for diagnostic features or select against non-diagnostic features (58). In situ 120 sequencing approaches may be another route to increased throughput once methods have 121 been industrialized. Finally, robust software pipelines will be needed that can rapidly analyze the 122 high-dimensional data and output perturbation landscapes that are able to diagnose disease. 123 Comparing human with mouse and other species can reveal the power and limitations of 124 model organisms for understanding human genotype to phenotype relationships. In many 125 cases, the same broad cell classes are found in mammalian tissues, and cell states can be 126 integrated across species (39). For example, recent single-cell analysis of the mouse kidney 127 suggested that most known kidney disease-associated genes in humans map to the mouse cell 128 counterparts(59). However there are cases in which the human and mouse tissues have 129 diverged significantly in terms of cell composition and gene expression. For example, humans 130 and other primates have a specialized area of the retina called the fovea, which can be 131 distinguished based on the particular proportion and types of retinal neurons that have distinct

132 expression signatures from other areas of the retina(60). Mice on the other hand lack a fovea

altogether, making it a poor model system for many blindness disorders. It may be possible to

134 leverage such differences to understand the underlying genetic mechanisms that control human

135 cell phenotypes. Altogether, comparisons between species, between human individuals, and

- between healthy and disease conditions can illuminate variety of human cell phenotypes, and
- help to link the genome to specific cell states. Now we need creative strategies to move past
- 138 correlation and establish the functional relevance of the observations from these highly-resolved139 maps.
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### 141 Stem cell and genetic manipulation tools

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143 There has been an exciting revolution in the stem cell biology field, which has made it possible 144 to generate diverse human cell types in controlled 2D cultures(61), and to generate complex 3D 145 tissues that resemble the primary tissue/organ counterparts (termed organoids)(62, 63). 146 Organoid protocols have been developed for various parts of the brain, liver, intestine, lung, 147 kidney, stomach, etc., and the protocols are being optimized for stereotyped morphology(64) 148 and inter-organoid reproducibility (65, 66). Many of these systems have been analyzed by 149 single-cell genomics and the data compared with their primary tissue counterparts to quantitate 150 how accurately cells states are recapitulated (67). The power of these in vitro systems is that 151 they are specific to an individual, enable replicate measurements over a time course, are 152 amenable to genetic manipulation and lineage recording in diverse environmental conditions, 153 could be used for high-throughput screening, and can recapitulate certain disease phenotypes 154 (Figure 3). For example, recent work showed that cerebral organoids recapitulate neuronal 155 migration defects observed in patients with periventricular nodular heterotopia, and single-cell 156 transcriptomics identified a perturbed differentiation trajectory (68). In addition, cerebral 157 organoids were used to identify specific cell states that were sensitive to hypoxia conditions that 158 occur in premature births, and organoids were used to screen for small molecules that 159 prevented loss of of these cell states(69). As detailed above, integrating multiple single-cell 160 genomic measurements together with cell history recorders and 3D spatial reconstructions will 161 soon provide very exciting high-resolution phenotyping strategies in these personalized models 162 of disease. 163 Culture systems that can recapitulate human development and physiology in vitro enable 164 researchers to use RNA-guided CRISPR-associated (Cas) nucleases to interrogate these 165 systems and link genotype to phenotype. CRISPR-Cas nucleases come in various natural as

166 well as synthetically engineered flavors enabling diverse genome and epigenome

167 modifications(70). These tools were originally established based on the Cas9 protein for gene

- 168 editing in immortalized mammalian cell lines(71-73) and then harnessed to link phenotype to
- 169 genotype using both a forward and reverse genetics approach (Figure 4). Reverse genetic
- approaches based on CRISPR, generally termed pooled CRISPR screens, involve the: i)
- 171 production of genome-scale or sub-genomic gRNA libraries; ii) low multiplicity of infection
- delivery to cells such that single cells receive single perturbations; iii) enrichment or depletion of
- cells based on a cellular phenotype of interest (e.g. proliferation, death, or presence/absence of
   selectable marker or reporter); and iv) identification and analysis of genes corresponding to
- 174 selectable marker or reporter); and iv) identification and analysis of genes corresponding to 175 enriched and/or depleted gRNAs. Pooled CRISPR screens were originally demonstrated in

176 human cancer cell lines (74, 75) but were recently extended and optimized in human iPSC 177 culture systems to identify genes that regulate pluripotency(76). Moving beyond gene knockout, 178 catalytically inactivated Cas proteins fused to effector domains enable diverse perturbations. 179 including transcriptional activation (CRISPRa) or inhibition (CRISPRi)(77), DNA methylation or 180 demethylation(78), histone acetylation(79), DNA (80) or RNA(81) base editing, as well as 181 others(82). Many of these RNA-guided Cas effector proteins have successfully been used in 182 pooled CRISPR screens further expanding our capacity to link diverse genetic and epigenetic 183 features to phenotypes (77, 83-85). Researchers have started to bring these technologies to 184 human iPSC cells by establishing stable cell lines that have inducible expression of the different 185 Cas effectors, and these lines can then be used to explore phenotype-genotype relationships in 186 a diversity of human cellular and tissue contexts. (5, 86, 87)

187 More recently, exciting work has merged CRISPR screening with single-cell genomic 188 readouts(88-91). In these methods pools of gRNAs are introduced into cells along with a Cas 189 protein such that cells express different gRNAs. Transcriptomes can be sequenced in single-190 cells and the gRNA that is expressed per cell can be determined based on an associated 191 barcode, or through direct sequencing of the gRNA. In this way, the effect of many different 192 gene perturbations can be examined in the same experiment with single-cell resolution. There 193 are several considerations when designing single-cell perturbation screens in organoids, and 194 optimizations on the initial protocols to reduce barcode recombination will enable more sensitive 195 and accurate readouts (92). One needs to determine how many genes can be targeted based on 196 a power analysis taking into account cell heterogeneity of the system (# cells), proportion of 197 mutant and wild-type cells, depth of sequencing, # reads per cell, effect size of perturbation, and 198 cost. Clonal selection within a stem cell culture or within an organoid can have an impact on the 199 results, especially if the organoid system is initiated from a composite of many different stem 200 cell clones (e.g. cerebral organoids). Cas protein can be constitutively or transiently expressed, 201 or induced through multiple strategies (e.g. Tet/On, Cre) and gRNAs can be introduced into the 202 cells or organoid through different delivery methods (AAV, lentivirus, transposon). Currently the 203 gRNA or barcode needs to be the read-out rather than the genomic lesion making the readout 204 correlative. If the proportion of mutant cells is too high, then the organoid may not develop 205 properly and the presence of a sufficient proportion of wild type cells could buffer mutant effects. 206 It is important to incorporate a selection feature of the cells receiving the gRNA (e.g. 207 Fluorescence). Finally, genetic perturbations might be cell autonomous or non-autonomous and 208 this can be difficult to distinguish in pooled screens with mosaic organoids, making arrayed 209 screening important alternatives. CRISPR/Cas9 screening based on single-cell sequencing in 210 iPSC-derived organoids has a rich future in the exploration of human cell phenotype to 211 genotype relationships. This will be made possible through innovations to increase throughput 212 to perform combinatorial genetic interaction and massively multiplexed screens, new 213 technologies to combine screening with molecular recording (7, 93) and lineage tracing, and in situ readouts(94) based on in situ sequencing technologies to open up exploration into spatial 214 215 effects of genetic perturbation. 216

217 Conclusions and outlook: In this review, we aimed to provide a foundational overview of the 218 current state of single-cell genomic-based phenotyping of human organs and organoids, and 219 how CRISPR-Cas technologies will enable phenotypes to be functionally linked to regions of the 220 human genome. We envision that the descriptive phase of single-cell genomics, where cell 221 phenotypes are catalogued for each healthy human tissue, will culminate in 4D resolved in silico 222 simulations that enable researchers to walk into the tissue, point to a cell at a location within the 223 tissue at a particular time point, and know its molecular features and its interaction with other 224 cells within the microenvironment. In the short term, the goal is to integrate and render different 225 feature (RNA, DNA, chromatin state, lineage) measurements into three-dimensional spatial 226 volumes with subcellular and temporal resolution. It will be exciting to incorporate single-cell 227 molecular measurements with in toto imaging of developing or cleared human organs/organoids 228 with cellular and subcellular resolution (95-98). Innovations in virtual-reality microscopy are 229 starting to bring together immersive visualization and simulation of imaged-based data with 230 hand gesture control, and the first virtual reality platform for the visualisation and analysis of 231 single-cell gene expression data has been developed(99).

232 Perturbation screens in human cells could be integrated with 4D spatiotemporal models 233 to weigh the functional relevance of genes and regulatory regions for establishing molecular, 234 cellular and systems-level phenotypes. However, there are still many limitations that require 235 innovations in the stem cell and organoid field that will enhance the biological insight that can be 236 attained from these efforts. Specifically, organoid morphology may not be stereotyped, there are 237 missing or off-target lineages, organoids are not integrated with other relevant organ systems, 238 and iPSC-derived organoids follow development and may not reflect processes in adults. It will 239 be important to continue to use single-cell genomics to assess the precision of novel organoid 240 protocols, and compare lineage and fate maps with other mammalian counterparts. 241 Establishment of stem cell resources from different populations (100) or the same individuals 242 from whom there are reference atlases will be useful for establishing a foundation for 243 quantitative comparisons across protocols. Furthermore, well characterized iPSC lines that 244 contain various flavors of CRISPR/Cas systems for genetic purturbation screens and cell fate 245 recording, together with a suite of diverse fluorescent reporters(101), will push the field forward.

246 Finally, a drive toward industrialization of single-cell sequencing pipelines on diseased 247 tissues and organoid models could bring exciting prospects for disease classification and 248 personalized medicine. This will require close collaborations between basic and clinical 249 researchers, as well as industry partners, to identify the unmet medical needs where single-cell 250 sequencing could have the most immediate effect. There are many disorders where multiple 251 genetic drivers are known, however it is increasingly clear that the different causative genes are 252 expressed in very different cell types(102). This is a major conundrum where molecular 253 dissection of the dissorder in primary tissues and/or organoid models could enable a refined 254 classification of the disorder, and also identify mechanisms that underly particular disease 255 presentations. Optimization of organoid protocols together with increased single-cell genomic 256 throughput would also enable testing disease-associated environmental conditions and potential 257 pharmacological-, gene- and cell-based therapies. There are many obstacles that remain, 258 however the field is moving forward at an extraordinary pace and it will be exciting to see where 259 it goes from here.

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### 263 **Figures** 264



#### 265

266 Figure 1: Human phenotyping in the single-cell genomics era. (a) Many different single-cell

- 267 genomic methods have been developed to profile cell heterogeneity in human organs across
- space and time. (b-c) These measurements can be integrated to build phenotypic maps that
- cross sub-cellular, cellular, and tissue/system scales.



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- 272 Figure 2: Human organ maps can resolve disease phenotypes. (a) Single-cell resolved
- 273 human organ phenotypic maps can be used to identify cell states that are likely most impacted
- by human disease. (b) In the future, diseases can be grouped into molecularly defined
- 275 subclasses based on single-cell genomic (SCG) phenotyping. Many obstacles remain for
- bringing SCG phenotyping directly to patients in a clinical setting.
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Figure 3: Human organoids to recapitulate human phenotypes in vitro. (a) Human

organoids derived from induced pluripotent or adult stem cells are genetically tractable and can

283 recapitulate complex tissue level phenotypes in controlled culture environments.

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**Figure 4: Genetic manipulation toolkit to link phenotype to genotype using stem cells.** A diverse array of genetic manipulation tools based on the CRISPR/Cas system can be deployed in organoids to test predictions from single-cell genomic surveys of organs and organoids and link genotype to phenotype. Shown are examples of potential CRISPR/Cas toolkit applications in human organoids.

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