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Mapping human cell phenotypes to genotypes with single-cell genomics

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ABSTRACT

The cumulative activity of all of our cells, with their myriad of interactions, life histories, and environmental experiences, gives rise to a condition that is uniquely human, and specific to each individual. It is an enduring goal to catalog our human cell types, to understand how they develop, how they vary between individuals, and how they fail in disease. Single-cell genomics has revolutionized this endeavor as sequencing-based methods provide a means to quantitatively annotate cell states based on high-information content and high-throughput measurements. Together with advances in stem cell biology and gene editing, we are in the midst of a fascinating journey to understand the cellular phenotypes that compose human bodies and how the human genome is used to build and maintain each cell. Here we will review recent advances into how single-cell genomics is being used to develop personalized phenotyping strategies that cross subcellular, cellular, and tissue scales to link our genome to our cumulative cellular phenotypes.

Phenotyping in the single-cell sequencing era

Phenotype can mean many things, but in general it is a way to classify a set of properties that arise from the interaction of an individual's genotype with its environment. It is a reductionist, yet powerful, approach to take human phenotyping down to the level of single cells and use molecular states within cells to establish phenotypes at the molecular, cellular, and tissue/system level. Single-cell sequencing technologies can measure thousands of individual features per cell for thousands of cells at a time, providing a quantitative and ultra-high resolution snapshot of cell and molecular states composing a human tissue, organ, or other biosystem. Currently, there are protocols available to measure the RNA content, DNA sequence and methylation status, chromatin structure and accessibility, and protein composition in single cells(1). In addition to molecular features, cell histories can be measured in certain scenarios where mutations have arisen in nuclear(2-4) or mitochondrial(5) DNA that distinguish lineages. Furthermore, in genetically tractable model systems (such as mice, zebrafish, and organoids), it is possible to record cell fate histories and infer lineage trees using reporter barcodes and genetic scarring based on RNA-guided CRISPR (clustered regularly interspaced short palindromic repeats)-associated (Cas) nucleases(6-9). So far, generally one or two single-cell measures have been used to phenotype individual cells within tissues in each experiment. However, the field is moving towards multimodal measurements from the same cell that capture

44 transcriptomic, genomic, epigenomic, and lineage states to enhance feature quantifications and
45 give a richer picture of a cell's phenotype at any given moment(10-12). These technologies are
46 forcing scientists to grapple with previous notions of "cell type", as cell properties can vary
47 discretely (type or subtype), as well as continuously (state), and classification may not always
48 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.

82

83 **Disease associated cell phenotypes**

84

85 As each human and mouse organ is mapped at single-cell resolution across space and
86 time, a next phase of inquiry is to understand how genetic changes impact human phenotypes.
87 Disease association studies have used naturally occurring 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
96 measures combining single-cell DNA, RNA, and chromatin can locate portions of the genome
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 RNAseq 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 miniaturized 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
133 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
136 between healthy and disease conditions can illuminate variety of human cell phenotypes, and
137 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-resolved
139 maps.

140

141 **Stem cell and genetic manipulation tools**

142

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
170 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
172 delivery to cells such that single cells receive single perturbations; iii) enrichment or depletion of
173 cells based on a cellular phenotype of interest (e.g. proliferation, death, or presence/absence of
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
214 situ readouts(94) based on in situ sequencing technologies to open up exploration into spatial
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

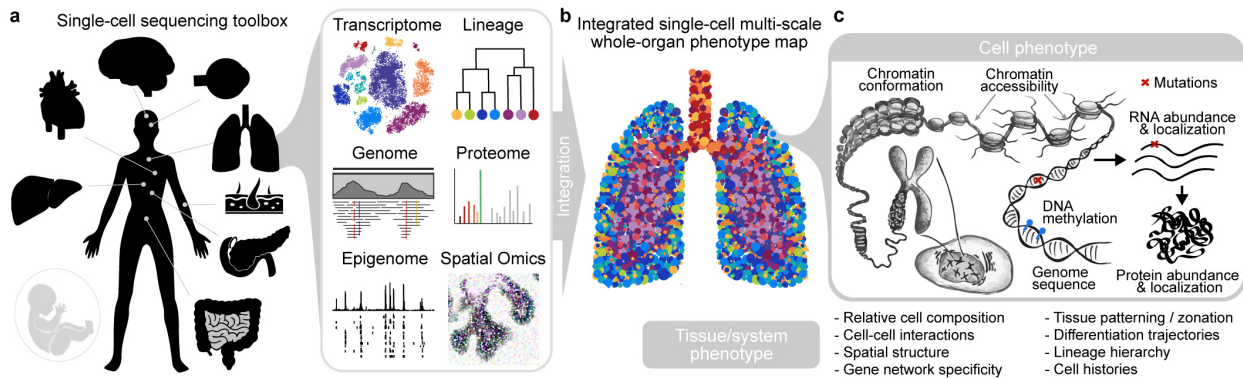
220 human genome. We envision that the descriptive phase of single-cell genomics, where cell
221 phenotypes are catalogued for each healthy human tissue, will culminate in 4D resolved *in silico*
222 simulations that enable researchers to walk into the tissue, point to a cell at a location within the
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 perturbation screens and cell fate
245 recording, together with a suite of diverse fluorescent reporters(101), will push the field forward.

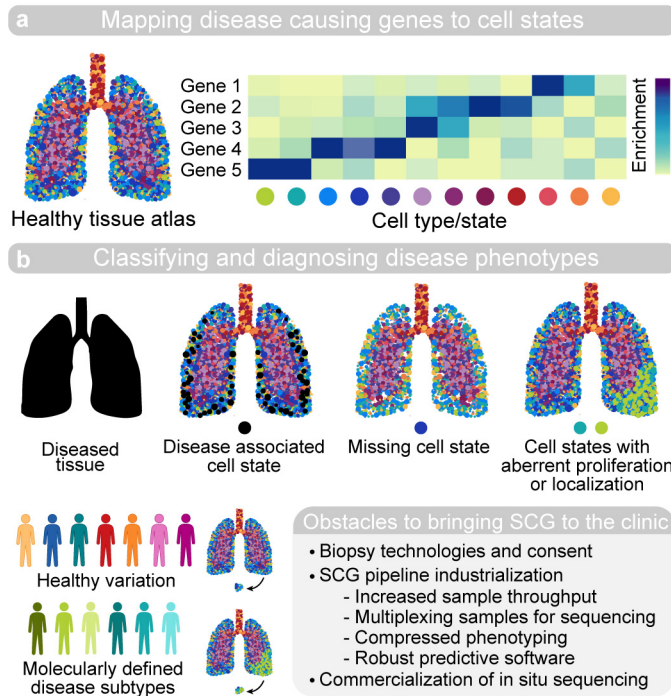
246 Finally, a drive toward industrialization of single-cell sequencing pipelines on diseased
247 tissues and organoid models could bring exciting prospects for disease classification and
248 personalized medicine. This will require close collaborations between basic and clinical
249 researchers, as 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 disorder 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

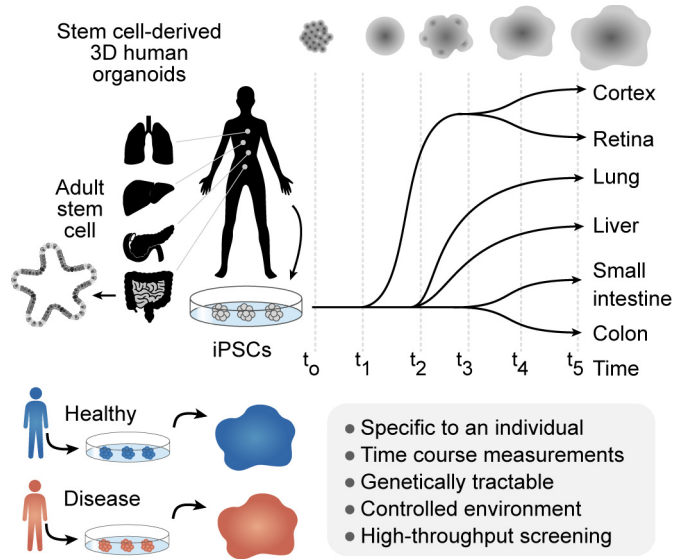


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 266 **Figure 1: Human phenotyping in the single-cell genomics era.** (a) Many different single-cell
 267 genomic methods have been developed to profile cell heterogeneity in human organs across
 268 space and time. (b-c) These measurements can be integrated to build phenotypic maps that
 269 cross sub-cellular, cellular, and tissue/system scales.



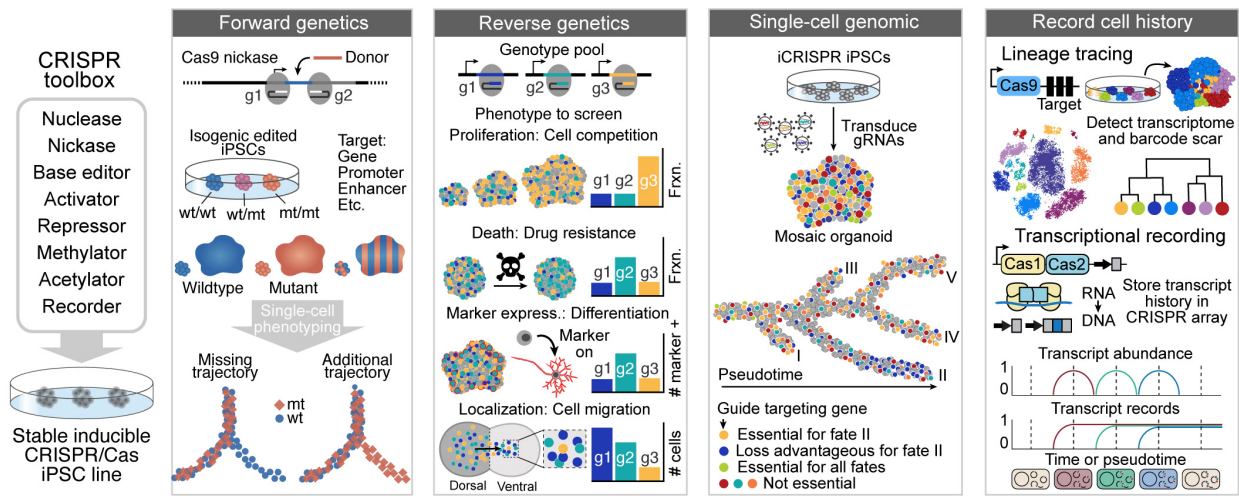
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 272 **Figure 2: Human organ maps can resolve disease phenotypes.** (a) Single-cell resolved
 273 human organ phenotypic maps can be used to identify cell states that are likely most impacted
 274 by human disease. (b) In the future, diseases can be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

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280
 281 **Figure 3: Human organoids to recapitulate human phenotypes *in vitro*.** (a) Human
 282 organoids derived from induced pluripotent or adult stem cells are genetically tractable and can
 283 recapitulate complex tissue level phenotypes in controlled culture environments.

284
 285



286
 287 **Figure 4: Genetic manipulation toolkit to link phenotype to genotype using stem cells.** A
 288 diverse array of genetic manipulation tools based on the CRISPR/Cas system can be deployed
 289 in organoids to test predictions from single-cell genomic surveys of organs and organoids and
 290 link genotype to phenotype. Shown are examples of potential CRISPR/Cas toolkit applications
 291 in human organoids.

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