engineered organotypic models: they do not reproduce themselves; many of the systems are assembled as artisan pieces with many parameters that can affect the model so it can be difficult to teach; many different biomimetic systems or variations would be expected to emerge to highlight different biological events and this customization inherently may limit wider adoption of each specific system; and it remains unclear which models scientists should congregate around versus leave under-investigated.

Despite these hurdles, the eventual incorporation of these synthetic biomimetic culture systems into biomedical research laboratories is inevitable. The confluence of technological advances in the engineering and biological communities appears to be a virtual perfect storm that will push us to continue establishing engineered 3D organotypic cultures. On the biological side, iPSC technologies and stem cell biology are coming together to advance access to human cell types and the application of genomic editing technologies offers the possibility of both modeling human genetic diseases and mechanistically implicating molecular players in these culture systems. On the engineering side, a suite of technologies have been established that can be used to build various types of system for organ-on-chip applications, including the development of biomaterials that can begin to mimic and decouple aspects of the ECM, the application of microfabrication and nanofabrication tools such as microfluidics to support cell-based systems, advances of 3D printing and other technologies to organize cells in three dimensions, microscopy advances to observe living cells in 3D contexts, and the use of insights gained by tissue engineers to assemble cells and ECM. The dire need for better models of human physiology and disease than either traditional cell culture or animals also provides a pull to advance these systems. Last, while ultimately these systems may become a primary platform for preclinical testing, their development will play a major role in our basic understanding of life’s design principles. Analogous to the in vitro reconstitution of subcellular processes, the iterative effort that leads to the synthetic reconstitution of multicell-type morphogenetic events will reveal the key components and subsystems necessary to stabilize within their surroundings and will at a minimum become a mainstay approach alongside standard reductionist and animal models to deepen our understanding of life.

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The Fuzzy Cell
Textbook views of cellular components, from protein complexes to organelles, follow the paradigm ‘localization = function’. If a protein is found at a cellular location it also functions there. Consequently, the focus of organelle proteomics has been to get the localization right. For decades this was attempted by subcellular fractionation and by sorting out assumed contaminants. However, protein location may have other reasons than function: cellular components possess an intrinsic, compositional ‘fuzziness’.

An often overlooked feature of subcellular organization is that it results from affinities and equilibria, in other words is quantitative and not qualitative. Membranes act as barriers but also need to be permeable. The nuclear envelope, for example, is permeable to proteins smaller than ~40 kDa. However, larger proteins might also make an uncontrolled entry into the nucleus, for example by having some affinity to the nuclear import machinery or at the end of mitosis, when the endoplasmic
The potential impact of dynamic equilibria is particularly obvious for the composition of non-membrane-enclosed compartments such as nuclear bodies and cytoplasmic granules. Proteins arrive there by diffusion and stay as a result of a preference for the environment of the respective compartment. However, it is highly unlikely that concentration gradients and local affinity will generate a binary sorting result, placing only proteins in those compartments that the cell needs to have there for functional reasons. For example, proteins appear not to exclusively localize to nucleoli, despite their enrichment there [4].

The cell does tolerate sorting noise. Possibly it is an essential part of evolution, allowing proteins to acquire a local function under some selection pressure. Proteins can acquire new subcellular localizations during evolution, as seen for duplicated gene pairs in yeast, which frequently possess functions in different organelles [5]. Proteins can also occupy multiple subcellular compartments as a result of their biosynthesis, transport, maturation, storage, or regulation. These processes are necessary for proteins to arrive in mature form at the location where they function. Consequently, transitory locations are ‘true’ locations of these proteins but not the sites of their function. Finally, multifunctional proteins exist that localize and function in multiple organelles, such as the mitochondrial prohibitins that ‘moonlight’ as nuclear transcription factors.

In the most recent draft of an organellar map of proteins it was noted that almost half of the observed proteins could not be assigned to discrete cellular locations [6]. Therefore, fuzziness appears to be a widespread phenomenon. If these proteins are to be placed onto a cellular map a different approach is needed. We propose a new concept to describe cellular organization, which combines indicators of protein function with localization data in a probabilistic framework.

A Potential Solution: Adding Function to Localization

Methods will have to be developed that can distinguish between proteins that function at a location and those that are present owing to biological leakiness or imperfections in purification. This requires spatial data (colocalization or co-fractionation) to be combined with sources of protein function. One potential way of achieving this is to use machine-learning algorithms to integrate a variety of data sources that include this information (Box 1).

Using this approach, many studies have built on mRNA or protein co-variation across multiple biological experiments as the source of functional data. To generate a compendium of mitochondrial proteins, mitochondrial fractionation proteomics has been combined via naïve Bayes machine learning with additional data, including mRNA coexpression and sequence features such as the presence of mitochondrial target peptides or characteristic protein domains [7]. For mitotic chromosomes, a combination of proteomic data and domain annotation was used to segregate putatively functional components from hitchhikers [1]. This led to the observation that function at a subcellular location can also be inferred from proteomics data alone. This follows a two-step procedure: first, proteins are quantified across multiple biochemical isolations of a cellular structure, obtained from different perturbed cells as starting material [3,8]. Second, one determines the co-variation of all identified proteins with known functional components of that organelle (Figure 1). Proteins with similar functions tend to behave more similarly to each other than to unrelated proteins across different biological conditions, for example in response to drug treatments or cell differentiation. The ‘behavior similarity’ or co-variation can be measured using multi-classifier combinatorial proteomics (MCCP) [1], which is based on another machine-learning approach, random forests. So far, both chromatin components [1,3] and mitochondrial proteins [8] can be determined on the basis of their co-variation, suggesting this could be a general method to determine functional organelle composition and an alternative to approaches based on co-fractionation. Indeed, co-variation was better suited to distinguish functional from non-relevant chromatin-bound proteins than classical, purification-based approaches [3]. Protein co-variation can also inform on organelle composition for organelles that contaminate the biochemical purification of another organelle [8].

In principle, the more different biological conditions that are tested for the composition
of an organelle, the better one can capture its constitutive, functional components. Importantly, instead of choosing an arbitrary cutoff to separate genuine organelle components from contaminants, machine-learning scores could be turned into a probabilistic version of gene ontology that fuses functional and localization considerations. A first example could be seen in interphase chromatin probability (ICP), possibly rephrasing ICPs as ‘integrated compartment probabilities’ [3] (Figure 1C). ICPs can be generated relatively easily for cellular structures of interest, provided that training sets and proteomics data for the species are available. The outcome is a list of all proteins detected in the analysis together with their probability of being a functional component of that organelle. An ICP of 0.8 predicts that 8 of 10 uncharacterized proteins with this value have a functional link to the organelle. One limitation of this approach is that it only works for organelles with sufficiently well-characterized components, although training sets do not need to be large because MCCP has been applied to protein complexes [9,10].

Application of Compartment Probabilities in Targeted Studies
ICPs are being applied. Proteomics experiments typically distinguish between relevant proteins and background through quantitative comparison. For example, DNA replication factors could be identified because they are enriched on replicating chromatin over mature chromatin. However, because these two chromatin states differ in their protein composition they also attract different background proteins [11]. Consequently, not all proteins that differ significantly between these two states are related to DNA replication. More than half of 1000 well-characterized proteins enriched on replicating chromatin were classified as biochemical contaminants because they were known to function elsewhere in the cell. This made it difficult to select candidates for novel DNA replication factors among the 300 co-enriched uncharacterized proteins. Filtering the dataset for proteins with high chromatin ICPs removed 90% of the contaminants, while retaining 90% of the known replication factors, and pinpointed 93 uncharacterized proteins as promising candidates for follow-up studies. Experimental validation for seven uncharacterized proteins enriched on replicating chromatin confirmed that three with high ICPs were indeed chromatin-based, and four with low ICPs were indeed background [11]. Likewise, ICPs guided the analysis of Cdk-dependent changes in S-phase chromatin. Of 114 proteins whose chromatin association was significantly and reproducibly dependent on Cdk activity, more than half were considered to be contaminants and 90% of these could be removed by ICP-based filtering [3]. Interestingly, the concept of protein covariation can also inform on the inner organization of organelles. For example, the relationship between protein complexes and novel complex components could be studied in the context of intact mitotic chromosomes [1,10].

Concluding Remarks and Future Directions
Not every cellular localization of every protein has a functional consequence, and we need tools that will allow us to disentangle those that do from those that do not. This will enhance our ability to study cellular processes, and will increase our appreciation and
understanding of the cell at a systems level. As more evidence for proteins existing in multiple cellular components accumulates, purely qualitative annotations will become more limited. Such annotation efforts have been essential for biological research in the past, but categorical annotation, without information on functionality for many proteins, risks becoming meaningless. While we currently have only acquired probabilities for chromatin- and for mitochondria-based function, future experiments will reveal the probability with which these and other proteins function in other organelles. Over time, it could lead to a quantitative, big-data-driven map of the cell, describing where each protein is present, and more importantly, where their functions are.

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