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PERSPECTIVE

In preprints: improving and interrogating embryo models

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How the complexity of an embryo arises is a question at the core of developmental biology. Key discoveries have been made by observing how embryos develop. In a complementary way, a recent bottom-up approach has harnessed the ability of stem cells to self-organize into structures resembling embryos at various stages. These embryo models go under different names according to the stages or tissues they mimic (e.g. blastoids, gastruloids, axioloids, trunk-like structures, etc.) and, when efficient, predictable and predictive of development, they expand our ability to investigate the path to embryo formation by overcoming technical and ethical issues associated with embryos. Here, we highlight two recent preprints that make important steps towards methods to improve and interrogate embryo models. The first preprint (Anand et al., 2022 preprint) tackles the problem of efficiency and reproducibility in an original way by smartly combining engineering with machine learning and, subsequently, interrogating the properties underlying robustness. The second preprint (Wehmeyer et al., 2022 preprint; now published in Development) cleverly shows how chimeric gastruloids built using fluorescently labelled cells carrying different genotypes produce new insights into well-hidden functions of classical developmental genes. Together, these two papers build a path toward making embryo models useful for discoveries.

In their preprint, Anand et al. propose a new approach to increase the efficiency and reproducibility of embryo models through a combination of engineering and machine learning. They build a human embryo model that mimics aspects of axial elongation by growing and differentiating pluripotent stem cells on coverslips previously imprinted with randomly positioned micropatterned islands of extracellular matrix. These micropatterns allow cells to be confined in colonies of specific sizes and geometries. The authors subsequently evaluate the influence of colony density on spontaneous axial elongation and polarization. Polarization is quantified by measuring the asymmetry of colonies by staining for CDX2, a marker of axial progenitors termed neuromesodermal progenitors (NMPs). As noticed previously (Blin et al., 2018), there is a certain variability in the polarization of colonies that is thought to originate from suboptimal boundary conditions. However, Anand et al. note that there is less variability between embryo models that occupy the same position in coverslips with identically distributed micropatterns. This suggests that the embryo models are somewhat coupled and can influence each other's ability to elongate. Accordingly, it has been reported previously that the geometric arrangement of multiple colonies affects their ability to pattern spontaneously (Blin et al., 2018). Anand et al. thus devise a strategy to leverage these observations in order to maximize the ability of colonies to elongate. To this aim, they collect data from thousands of embryo models and run machine learning algorithms to predict their polarization given the local geometric and spatial arrangements of micropatterns. This allows them, using computer simulations, to identify the arrangement that maximizes polarization, namely six embryo models arranged in hexagonal patterns spaced by 1.6 mm.

In the next step, they make use of their beautifully optimized model to look for mechanisms underlying axial elongation. Through single-cell RNA sequencing and subsequent validation, they observe patterned expression of WNT5A, PRICKLE1 and FGF8 in the posterior region. By treating cells with IWP3, which inhibits porcupine O-acyltransferase, and hence WNT secretion, either at the beginning or halfway through the experiment, they see that WNT secretion is not necessary for the initial specification of $CDX2^{+}$ cells, but is continuously important for the elongation process. Live imaging suggests that elongation occurs by WNT-dependent directed cell movements along the axis that are not due to a directionality of the planes of cell divisions. Through genetic engineering, they further find that the expression of CDX2, which is known to control WNT ligand expression (Chawengsaksophak et al., 2004), is necessary for elongation. Because elongation is also known to rely on FGF ligands, they test the impact of a MEK inhibitor (PD0325901) and show that this also prevents elongation. Interestingly, a pulse of FGF2 is sufficient to sustain elongation even in the absence of the canonical WNT activator CHIR99021 as it helps maintain NMPs, a phenomenon that correlates with the expression of TBXT. Given that TBXT is known to drive WNT and FGF ligand production, a feed-forward loop is likely to occur wherein WNT and FGF expressed by NMPs induce the expression of more ligands, thus sustaining tissue elongation. Hence, the authors propose that the system is excitable and entrains its own elongation. However, such excitable systems are known to be susceptible to noise and thus prone to errors. How can elongation happen in such a stereotypic way without making mistakes? Why does this stereotypic behaviour occur only when embryo models are placed at a certain distance from one another? It turns out that inhibitors of the same WNT pathway, namely SFRP1 and SFRP2, are secreted in a gradient anti-correlating the WNT one. Interestingly, when SFRP1 and SFRP2 are genetically ablated, the embryo model forms multiple NMP domains instead of one. This suggests that SFRP1 and SFRP2 are redundantly acting WNT inhibitors expressed away from the NMPs to guide the inherently fluctuating excitable systems and prevent the appearance of multiple domains. This approach is innovative, systematic, and proves useful for understanding how robustness originates from precise geometric constraints channelling intrinsic self-organized loops that are inherently prone to noise disturbances (Murray, 2013).

Embryo models also lend themselves to more complex and potentially more informative manipulations. For instance, *in vitro* models can be formed with a mix of cells with different engineered

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genotypes, an operation that remains complicated with embryos. Such mixes can be useful, for example for assessing the role of intrinsic genetic programmes (e.g. Hox collinearity; Beccari et al., 2018), cellular signalling interactions (e.g. tissue inductions; Vrij et al., 2022) or the mechanisms underpinning cell–cell competition (e.g. NFKB; Lima et al., 2021). Although this idea has already been applied to build chimeric embryos, *in vitro* models offer a more tractable system in which cellular composition can be finely tuned and single cells can be imaged and tracked more easily.

In a proof-of-principle application, Wehmeyer et al. (2022 preprint) show how chimeric gastruloids can be used to disentangle the cell-autonomous from non-cell-autonomous functions of classical gastrulation genes. They build chimeric gastruloids composed of wild-type (WT) cells mixed with cells carrying loss-of-function or inducible alleles for *Eomes* and brachyury (*T*), which encode two transcription factors that are crucial for cell differentiation during gastrulation. Thanks to fluorescent labels, cells are tracked to reveal the influence of genotype on their position, their fate and, more importantly, their impact on neighbours. For example, Wehmeyer et al. mix WT cells with cells harbouring a doxycycline-inducible expression cassette for a GFP-tagged *Eomes*. Forced expression of *Eomes* in a subset of cells cell-autonomously instructs domains of beating cardiomyocytes in ∼30% of chimeric gastruloids, which are very rarely present in gastruloids built only from WT cells. Mixing genotypes can thus add signalling or functional domains to embryo models in order to test their impact (Xu et al., 2021).

In another experiment, Wehmeyer et al. generate chimeric gastruloids by mixing WT and $T^{-/-}$ cells. Similar to embryos, gastruloids fully depleted of *T* cannot elongate. However, by mixing 10% WT and 90% $T^{-/-}$ cells, the authors observe that *T* not only remains off in the knockout cells, but also ceases expression prematurely in the WT cells. This suggests a non-cell-autonomous effect of *T*, in agreement with previous studies showing that Nodal expression (which induces brachyury expression) is first maintained by its own expression mediated by an asymmetric enhancer and is secondly enhanced by a cascade of BMP4 and activating Wnt3a, mediated by a proximal epiblast enhancer (Plouhinec et al., 2022). To investigate the cell-autonomous effects of *T* expression, Wehmeyer et al. decrease the proportion of $T^{-/-}$ cells to 10% and analyse the spatial distribution of the mutant cells. They observe that $T^{-/-}$ cells in the resulting chimeric gastruloids have specific location and fate biases: they are mainly located in the mid and posterior region of the gastruloids, where they tend to differentiate towards a definitive endoderm or a neuroectodermal fate, respectively. This shows how a specific genotype can direct cells into specific fates and suggests that the gene product of *T* can counteract these lineage-commitment programmes. Overall, this preprint shows how chimeric gastruloids are versatile tools that can generate embryo models with an increased repertoire of cell types and be used to probe the functions of genes during development at different levels.

The refinement of embryo models makes them more manipulatable, reproducible, efficient, and thus useful. How can embryo models best be used to study development? What level of fidelity is required? What can we learn from models that escape the route shaped through evolution? In our opinion, the two preprints presented here introduce experimental and computational approaches that will shape the predictive levels of embryo models and their capacity to answer new questions. As Anand et al. (2022 preprint) show, machine learning offers an efficient and targeted way to optimize protocols, while providing information on the interplay between geometric constraints and excitable molecular loops that affect axial elongation. Such information could, in turn, be tested in physical models the predictions of which can be quantitatively validated using mixed genotypes, as in chimeric gastruloids (Wehmeyer et al., 2022 preprint). Moving forward, this will help reveal the principles underlying the organization of complex tissues, organs and embryos.

Note added in proof

Wehmeyer et al., 2022 has now been published as: Wehmeyer, A. E., Schüle, K. M., Conrad, A., Schröder, C. M., Probst, S. and Arnold, S. J. (2022). Chimeric 3D gastruloids – a versatile tool for studies of mammalian peri-gastrulation development. Development 149, dev200812. doi:10.1242/dev.200812

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Competing interests

N.R. is an inventor on the patent ‘Blastoid, cell line based artificial blastocyst’ (EP2986711) and on the patent application ‘Blastocyst-like cell aggregate and methods’ (EP21151455.9), both maintained by the Institute for Molecular Biotechnology, Austrian Academy of Science.

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