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Single cells get together: high-resolution approaches to study the dynamics of early mouse development

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Abstract

Embryonic development is a complex and highly dynamic process during which individual cells interact with one another, adopt different identities and organize themselves in three-dimensional space to generate an entire organism. Recent technical developments in genomics and high-resolution quantitative imaging are making it possible to study cellular populations at single-cell resolution and begin to integrate different inputs, for example genetic, physical and chemical factors, that affect cell differentiation over spatial and temporal scales. The preimplantation mouse embryo allows the analysis of cell fate decisions *in vivo* with high spatiotemporal resolution. In this review we highlight how the application of live imaging and single-cell resolution analysis pipelines is providing an unprecedented level of insight on the processes that shape the earliest stages of mammalian development.

Keywords

Systems biology; quantitative high-resolution imaging; genomics; single-cell analysis; mouse embryo; lineage specification

1. Introduction

The development of an entire adult organism from a single fertilized egg is arguably one of the most complex and fascinating processes in biology. Its study necessarily involves the simultaneous consideration of several levels of organization, from the molecular through the cellular and tissue level, over a temporal scale. Breaking down a system into isolated modules for its study is experimentally practical and a good starting point. However, cells in culture or isolated genetic pathways cannot account for the temporal and spatial coordination of factors that guide the developmental process. More importantly, biological systems have emergent properties that do not exist within their isolated parts, and can only be revealed when studied as a whole. Thus, only a holistic, systems-level approach will provide insight into the complex genetic, molecular and physical processes that drive organismal development.

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Systems-level approaches apply high-resolution, single-cell analyses and take advantage of high-throughput data obtained from the parallel processing of large numbers of samples and targets. The early mouse embryo, especially at preimplantation stages, is particularly well suited for a systems-level analysis due to: (1) its stereotypical morphology and small size, which allows the study of most, if not all cells in the embryo, and their dynamic interactions with one another; (2) cellular processes that can be imaged at high-resolution [1–3]; (3) *in vitro* culture with no detriment to viability [4]; and (4) experimental access to individual cells while providing a relevant context for these cells — signals, positional and temporal information [5–7].

Over the last two decades the study of early mouse development has benefited from the development of novel imaging and genetic tools, the advent of genomic analysis methods, such as next generation sequencing (NGS) and an increase in the computational power available on desktop machines [8–10]. In this review we will rationalize the need for a systems-level approach for studying mammalian preimplantation development and discuss recent progress, emphasizing how current technology is facilitating the study, *in situ*, of cellular events that had previously been inaccessible.

2. The first steps to making a mouse

The preimplantation stages of mammalian development cover the period between fertilization and the implantation of the embryo into the uterus (4.5 days post-fertilization (E4.5) in the mouse) (Figure 1A). During this period, a series of sequential cell divisions of the zygote give rise to the morula, which in turn undergoes several morphogenetic changes to become the blastocyst. The blastocyst comprises two epithelial layers (the trophectoderm (TE) and the primitive endoderm (PrE)) that enclose a pluripotent cell population, the epiblast. The TE and PrE are extraembryonic lineages that support the growth of the epiblast into the embryonic ectoderm, which will give rise to the three germ layers during gastrulation (). After implantation, the TE will develop into the extraembryonic ectoderm (ExE) and the ectoplacental cone (EPC), which will give rise to the fetal part of the placenta. The PrE, on the other hand, develops into the endoderm of the visceral and parietal yolk sacs (VE and ParE, respectively) and contributes to the gut endoderm [11,12]. More detailed reviews on the molecular and morphogenetic events taking place during early mouse development can be found elsewhere [13–17].

The process of blastocyst formation is a paradigm of self-organization, where morphogenesis and cell differentiation take place independently of the maternal environment and generally do not involve maternal determinants [18]. Instead, lineage specification is the result of cellular interactions and the relative position of cells within the embryo. At the 8-cell stage (E2.5), the development of intercellular junctions between blastomeres results in compaction of the embryo and the creation of a physical constraint for cellular organization [2,19–21] (Figure 1A). Confined by this spatial restrictions, new cells generated by cell division are forced into either an inner position or an outer layer [1,3,22,23]. Outer cells retain a distinct apical domain on their exposed surface and consequently inhibit the Hippo pathway to activate a TE-specific genetic program. On the

other hand, inner cells, subject to symmetric cellular contacts and Hippo pathway activity, go on to form the inner cell mass (ICM) [24–34].

Over the ensuing 24h, signaling and intercellular interactions trigger the differentiation of epiblast and PrE. A subset of ICM cells produce and secrete fibroblast growth factor 4 (FGF4) [35,36], thus acting as sources of this signal. Although it has been proposed that FGF4-producing cells give rise to the epiblast and those that receive the signal become PrE, gene expression profiling has suggested that FGF4 may also play a role in epiblast development [36]. Nonetheless, this asymmetric signaling results in the differential activation of a receptor tyrosine kinase (RTK)-MAP kinase (MAPK) axis and the differential activity of the transcription factors NANOG and GATA6 — which mark the epiblast and the PrE, respectively [37–46]. These two cell types arise scattered throughout the ICM and subsequently become rearranged into two coherent and spatially segregated populations through a combination of cell movement and differential perception of positional cues (Figure 1A) [47–50]. PrE cells, which are capable of polarizing, accumulate at the interface with the blastocyst cavity, whereas epiblast cells migrate towards or remain on the inside of the ICM [47,48,50,51] (Figure 1A).

Cell specification in the early mouse embryo is thus driven by a combination of physical (spatial confinement, cell contacts), chemical (growth factors) and temporal factors, rather than by inherent pre patterning or a stereotypical cell division pattern. Therefore, these processes can only be fully understood when studying them within the context of the embryo with a systems perspective.

3. Why a systems approach to study the biology of the blastocyst?

Embryos are heterogeneous, dynamic, multi-dimensional entities. As such, it is desirable to analyze individual parts while taking into consideration their spatiotemporal position within the system. Such an approach allows, for instance, the identification of distinct cell behaviors over time (Figure 2A) and the quantification of heterogeneities in gene expression (Figure 2B), neither of which can be revealed by population-level analysis. However, systems approaches face a number of technical challenges, namely the amount of material (i.e., cells) available for analysis, the simultaneous quantification of the expression of multiple genes, and the integration of gene expression with positional information and cell behavior over a temporal scale.

Stem cells derived from embryos — such as embryonic stem cells (ESCs), trophoblast stem (TS) cells or extraembryonic endoderm (XEN) stem cells [52–56] — provide large amounts of cells for systems-level analyses and have been instrumental for interrogating the genetic and molecular pathways driving cell fate in the embryo [26,57–63]. However, these *in vitro* paradigms are poor surrogates to fully understand embryo development, as they lack the cellular heterogeneity and spatial organization of the embryo (different cell types organized in a dynamic three dimensional structure) and are not subject to the temporal constraints that cells in the embryo are (timeframe of developmental progression). Therefore, experimental efforts have focused on capturing temporal changes in gene and protein expression, position and identity in individual cells within the context of the embryo. In the following sections

we will discuss the contribution of these technical advances to our current understanding of early mammalian development.

4. Technical advances in single-cell level analysis

The techniques that facilitate single-cell level analyses of embryonic development can be grouped into two categories: (A) gene expression profiling (such as quantitative PCR (qPCR), microarray and mRNA-sequencing (RNA-seq)) and (B) *in situ* detection of mRNA and protein expression (such as fluorescence *in situ* hybridization (FISH), immunofluorescence and live imaging using fluorescent reporters). The former offer the highest throughput gene expression data at the expense of spatial and temporal data (Figure 2C). They allow quantitation of mRNA species present in individual cells, and the identification of cell populations based on the expression of multiple markers. However, they require the disaggregation of the tissue with the concomitant loss of information about relative cell position and cellular niche. On the other hand, *in situ* techniques, while offering more limited gene expression data, provide information about absolute and relative cell position, cell proliferation, protein levels and cell morphology. Furthermore, when applied to live samples, they afford the possibility to follow changes in gene expression while tracing individual cells and lineages over a temporal scale (Figure 2D).

Gene expression profiling has required the adaptation of protocols to allow the analysis of picogram amounts of material obtained from single cells [64–66]. These protocols have been successfully applied to both microarray [36,67] and RNA-seq [68] analyses of single cells from preimplantation mouse embryos. In addition, they have recently benefited from the application of microfluidics technologies, which streamline the preparation and analysis of nanoliter-scale samples [35,69]. While RNA-seq provides the highest resolution, it also generates larger volumes of data than microarrays [70] and therefore the choice of approach will depend on the needs of the study. When not searching for novel targets, lower resolution techniques, such as qPCR arrays can produce insightful data at a lower processing cost [35,69,71].

While *in situ* techniques cannot offer such a detailed picture of gene expression as profiling techniques, they allow the analysis not only of mRNAs, but also proteins, with much higher temporal and spatial resolution. Moreover, by harnessing the power of mouse genetics, expression of RNAs and protein localization in individual cells, as well as cellular behavior, can be tracked over time [2,7,47,72–75]. Improvements in these techniques have come from both the development of more sensitive detection reagents and the widespread use of high-resolution optical microscopy and image analysis methods. Besides commercial packages, individual labs are routinely developing their own image analysis tools, ranging from open source platforms, such as FARSIGHT (http://www.farsight-toolkit.org/wiki/Main_Page) or ImageJ (<http://imagej.nih.gov/ij/index.html>), to applications tailored for particular tasks or experiments [76,77].

This type of data is inherently quantitative, and the use of these techniques has resulted in a shift towards quantitation in mouse embryology studies, from early efforts in manual cell counting [29,47,78,79] to our current ability to analyze mRNA and protein expression in

virtually every cell in any given embryo [35,36,45,68,69]. The application of multivariate statistics, such as principal component analysis (PCA) or algorithms for clustering analysis, is necessary to understand these data [35,36,69,71], as well as the use of tools for the definition of gene pathways and networks and for gene ontology (GO) analysis [63,68,80]. The combination of these data with the ability to track events temporally with cellular and sub cellular resolution are allowing us to better understand the dynamic events that characterize embryonic development. Below we highlight examples of how the combination of both high throughput and computational analyses is already providing an unprecedented insight into the events that shape the mammalian embryo at the molecular and cellular level.

4.1. Transcriptional profiling offers high throughput data of few dimensions

It is currently possible to carry out single-cell resolution, transcriptional analyses on early mouse embryos using microarray [65,67] or RNA-seq [66,68]. In a recent study, Ohnishi *et al* [36] applied a single-cell microarray analysis to study the changes in gene expression that take place in *Fgf4* null preimplantation embryos. FGF4 is the instructive signal for PrE specification during mouse preimplantation development, and in its absence, all ICM cells retain expression of the pluripotency markers NANOG and OCT4 [37,38,41,43,44]. Building on previous methods for mRNA amplification [65], a higher-resolution picture of the transcriptional changes that take place in ICM cells as wild-type and *Fgf4* mutant blastocysts develop was generated. These data identified FGF4 as the earliest gene to show differential expression among ICM cells, thus predicting a divergence between epiblast (FGF4 positive) and PrE (FGF4 negative) populations [36]. Importantly, this study showed that *Fgf4* null ICM cells do not exhibit an epiblast-like expression profile. Although *Nanog* expression is upregulated in *Fgf4* mutant ICM cells and PrE marker expression is concomitantly downregulated, as previously observed [37,38,41,43,44], principal components analysis (PCA) mapped gene expression profiles of mutant ICM cells closer to those of earlier wild type epiblast stages. This finding indicates that epiblast development in *Fgf4* mutants is delayed, and suggests an additional role for FGF4 (or a non-cell autonomous role of the PrE) in epiblast development [36].

Another alternative approach involves the analysis of a defined set, or array, of genes using qPCR. The advent of nanoliter-scale, microfluidics-based platforms has been instrumental for this kind of approach, as they improve the sensitivity of the assays and allow to maximize the number of reactions performed on each single-cell sample [81]. First used to define changes in gene expression at sequential stages of preimplantation embryo development [35], this platform has been recently applied to the study of the epigenetic changes associated with the progression from oocyte to blastocyst [69]. In this study, Burton and colleagues focused their analysis on the earliest stages of development (from oocyte to the 32–64 cell stage) with an emphasis on chromatin modifiers. They showed that over the stages prior to blastocyst formation (oocyte to 8-cell stage) — and thus, before significant morphological changes have taken place and lineages are specified — individual cells cluster by developmental stage based on their expression of epigenetic modifiers alone. In contrast, differences in transcription factor expression at these stages were negligible, therefore indicating that over this period cellular states are defined by the profound epigenetic changes triggered after fertilization — associated with zygotic genome activation

— and not by lineage-specific genetic programs [69]. These findings provide insight into how epigenetic remodeling during the earliest stages of mouse development may be critical to the establishment of a suitable landscape for transcription factor-driven lineage specification in the blastocyst.

qPCR arrays combined with RNA-seq and single-cell functional assays were also recently applied to precisely pinpoint the embryonic origin of ESCs [71] and to assess the developmental potential of subpopulations of ESCs [82]. Comparative analysis of gene expression at sequential blastocyst stages and hierarchical clustering demonstrated that ground-state ESCs are most similar to the epiblast of E4.0-E4.5 blastocyst [71]. Supporting this notion, almost all epiblast cells (but none of the PrE cells) from these embryos were able to give rise to ESC lines when dissociated and cultured individually [71]. In another study, ESCs cultured in different conditions were shown to contain subpopulations defined by distinct gene expression patterns [82]. These expression patterns were associated with different developmental potential in chimera assays — where single cells were grafted onto a morula-stage embryo. These experiments revealed that addition of LIF to the culture media promotes the expansion of a totipotent subpopulation of ESCs [82]. These approaches thus have allowed to precisely define the *in vivo* counterpart to ESCs and to establish functional relationships between *in vitro* and *in vivo* pluripotent populations.

While whole transcriptome profiling techniques are able to provide a high-resolution picture of gene expression in single cells, they do so at the expense of other layers of information, such as developmental stage or positional data (Figure 2C, D). Embryo staging can be done accurately prior to expression profiling through fixation and staging of littermates of the embryos disaggregated [35,36]. By contrast, although attempts at preserving some degree of spatial information have provided insightful data [36,83], for the most part these approaches fail to capture absolute or relative cell position within the embryo and are incompatible with the analysis of temporal dynamics and lineage tracing.

4.2. In situ detection gives up resolution for more layers of information

In contrast to the tools described in the previous section, *in situ* detection techniques preserve the integrity of the sample and afford to place events on a temporal scale (Figure 2D). Highly sensitive probes and antibodies allow the detection of transcripts and proteins with cellular and sub-cellular resolution [37,74,84,85]. Similarly, nuclear-localized, bright, single-cell resolution fluorescent reporters developed over the past two decades, now allow live imaging and tracking of individual cells and cellular structures [2,3,8,86,87]. However, equally important, are the use of high-resolution optical imaging modalities, such as laser scanning confocal, spinning disc, multi-photon or light-sheet microscopes. Laser scanning confocal microscopy (LSM) offers the highest level of x-y resolution, and is currently the preferred method to study protein distribution in fixed samples. Spinning disc, multi-photon and light-sheet (or single plane illumination microscope, SPIM) microscopes, while offering lower x-y resolution, cause significantly less photodamage of the sample and offer an advantage for live imaging studies. Spinning disc and light-sheet systems offer greater speed of acquisition, and have been implemented for some studies, but they do not afford themselves as readily to multi-spectral imaging. Sample mounting, especially in the case of

mouse embryos, has also an issue with light-sheet systems. Multi-photon microscopes yield deeper (z-axis) tissue penetration than LSMs - although their higher cost and lower x-y resolution, currently make LSMs the tool of choice for most preimplantation embryo analyses. Coupled with advances in data acquisition are developments in image processing software and computational tools for image analysis and quantitation, which facilitate unbiased approaches to studying spatial configuration and protein abundance. In recent years, these developments have enabled a shift towards extracting quantitative data from immunofluorescence images and time-lapse movies of mouse embryos [47,75,80,88–91] (Figure 3).

A major hurdle to image analysis at preimplantation stages is the close proximity of individual cells; high cellular (or nuclear) density poses problems for automated nuclear segmentation. The image segmentation algorithm MINS (Modular Interactive Nuclear Segmentation) [76], has been recently developed to accurately and automatically segment nuclei and quantify fluorescence intensity in images of preimplantation embryos and ES cell colonies. This tool has been applied to study the role of the transcription factors OCT4 and GATA6 during preimplantation development [45,89]. The analysis of *Gata6* mutant embryos revealed that GATA6 is necessary for PrE specification, and that its absence cannot be compensated by addition of FGF4, thus demonstrating that GATA6 is necessary for the activation of the PrE program by FGF4 signaling [45]. Importantly, the use of MINS on immunolabeled embryos allowed the quantification of protein concentration in the nuclei and relative position at the single-cell level, which is not possible with traditional biochemical assays. Unexpectedly, this revealed that NANOG expression is not elevated in *Gata6* mutants, suggesting that GATA6 is not responsible for NANOG repression. Furthermore, protein quantification showed that GATA6 levels are reduced in *Gata6*^{+/-} embryos, which causes delayed PrE specification and, ultimately, a reduction in the size of the PrE compartment [45]. Of note, single-cell level quantitation of relative nuclear concentrations of proteins allows an unbiased assignment of cell identities *in situ*. Analysis of the relationship between cell position and cell identity revealed that cell specification in the ICM is initiated independently of either absolute cell position within the embryo or the relative location of cells to one another; this stochastic pattern is only resolved gradually into coherent populations as development proceeds [45].

4.3. Live analysis places events over a temporal scale

In addition to spatial information, *in situ* analyses provide a means to explore the temporal dimension, arguably a central feature of developmental processes. By using lineage-specific, fluorescent transcriptional reporters, we can study gene expression coupled to cell movement, cell division and cell death. For instance, this approach allowed the determination of the dynamic processes that lead to the formation of a coherent PrE layer from a dispersed cell population [41,47,49,50] (Figure 1A). It was found that active migration, combined with the perception of positional cues, segregates PrE and epiblast cells into two separate compartments [47,49]. Live imaging of a nuclear-localized reporter of *Pdgfra* expression (an early PrE marker) [92], revealed that changes in cell position influence both gene expression and cell survival [41,47,50]. Furthermore, fluorescent

reporters also allow the isolation of distinct subpopulations of cells for their characterization, using transcriptome analysis, chimera generation or *in vitro* assays [5,71,82,83,93].

Since the discovery of the importance of NANOG for the establishment of pluripotency, a number of studies have focused on determining its role in epiblast development *in vivo* [39,40,59,60,94]. Analyses of *Nanog* expression in ES cells using cytoplasmic GFP-based reporters and fluorescence-activated cell sorting (FACS) concluded that *Nanog* expression oscillates in ES cells and that its expression level determines cells' propensity to differentiate [94,95]. Although it has been suggested that a similar mechanism operates in the embryo, the answer to this question can only come from studying *Nanog* dynamics *in vivo*. Live imaging of a nuclear-localized *Nanog* transcriptional reporter has showed that, contrary to observations in made in ES cells, *Nanog* expression does not oscillate in the embryo [7]. Xenopoulos *et al* applied MINS [76] to segment individual cells and accurately track spatiotemporal changes in *Nanog:H2B-GFP* expression [7]. This analysis revealed epiblast cells maintain steady, high levels of the reporter whereas it is down regulated in cells committing to a PrE fate. It also showed that no cell-fate switches occur *in vivo* — once epiblast cells become specified in the early blastocyst, they do not convert to PrE, in agreement with previous studies [5,7]. The differences between these *in vivo* results and those obtained with ES cells may be explained by the differences between both systems. While ES cells are maintained in culture indefinitely, embryos are subject to tight temporal constraints, whereby epiblast and PrE lineages need to be established by the time of implantation. Therefore, even if *Nanog* expression were to fluctuate *in vivo*, the narrow time window for lineage specification would not allow these fluctuations to be observed. Alternatively, these differences may be due to differences between culture conditions used for ESCs and embryos or be the consequence of ESC-specific properties that emerge from their adaptation to *in vitro* culture.

Nuclear reporters can also be used to study developmental events beyond cell movement and gene expression changes. For instance, apoptosis or cell cycle dynamics can only be accurately assessed in live samples. Both ubiquitous and tissue-specific transcriptional reporters also allow the visualization of cell death events (which involve nuclear fragmentation) and their location within the spatiotemporal map of the embryo [6,7,47,96]. Nuclear-localized transcriptional reporters can also be used to study cell division and cell cycle dynamics, although tools tailored for the study of cell cycle dynamics, such as the Fucci system [97], are more powerful and are beginning to be applied to the study of mammalian development [98].

Finally, looking at subcellular resolution, live imaging can be used to study changes in protein and mRNA distribution within cells. Provided they do not interfere with protein activity, transcription factor fusions to fluorescent proteins have great potential to yield new insight into their DNA-binding dynamics *in vivo*, as recently shown for OCT4 in morula stage embryos [72]. Although less widespread, systems to visualize changes in mRNA localization and abundance in live cells exist [74,85,99], their application to the live analysis of gene expression in embryos will undoubtedly be feasible soon.

These studies show how surrendering some degree of throughput in favor of spatial and temporal information can unveil mechanistic details that cannot be inferred from looking at high-resolution, but temporally static pictures of individual cells. Moreover, they place events on the temporal scale, an essential consideration to understand the developmental process.

5. Future challenges

A number of key technological developments over the past decade have fueled a renewed interest in assessing the processes that shape the early mouse embryo. Current challenges include data analysis, the integration of single-cell resolution expression data with positional information and mathematical modeling.

The extraction of meaningful data from large datasets poses substantial challenges. On the one hand, the processing of large volumes of multi-dimensional data demands the application of multivariate statistics and involves computational knowledge beyond that of the traditional biologist. As a result, fruitful collaborations have been established between biologists, mathematicians and statisticians to tackle the challenge of data analysis. On the other hand, the processing of large volumes of data can easily become the limiting factor in terms of time, more so than the data collection itself, a factor that should be taken into consideration during the experimental design. Collaboration between biologists, bioinformaticians, statisticians and mathematicians, and an effort from all parts involved to understand all aspects of the process, is already allowing us not only to extract more information from these datasets, but also to reach novel conclusions that would not have been possible using classical analyses.

High-resolution studies produce very detailed information, making it hard to manually interconnect all the elements involved in the networks formed at all hierarchical levels. To adopt a systems-level approach where all layers of regulation are considered, mathematical models become necessary. Mathematical models provide an avenue to integrate the many factors that comprise a system — genetic, physical and temporal —, in a way that allows their role to be deciphered and to make testable predictions about the system's response to changes [100,101]. When it comes to understanding the dynamics of a system, descriptive models are useful, but they rely on empirical data that needs to be collected beforehand [46]. Predictive models, on the other hand, allow the testing of hypotheses and provide potential experimental outcomes that aid both in experimental design and data interpretation [23,101]. Attempts at modeling blastocyst formation that integrate mechanical forces operating in the embryo with changes in gene expression and cell position have already generated insight on the processes that determine cell fate decisions *in vivo* [23,102].

Finally, the biggest challenge with the present methods is to combine high-throughput expression data with spatial and temporal dimensions [100]. Current attempts at doing so [36,83], albeit limited in their spatial resolution, are encouraging. The use of combinatorial, multi-colored Rainbow cell labeling and imaging [93,103] prior to cell disaggregation — and ensuing loss of positional information — would give an opportunity to label many individual cells and retrospectively map them to their recorded location in the embryo.

Alternatively, live imaging the expression of multiple genes — despite its own challenges — will enable a more accurate determination of cell identities *in situ*. The use of other labeling methods, such as molecular barcoding [104], albeit not currently suitable for live imaging, would nevertheless allow retrospective clonal and lineage analysis. An added challenge in this respect is the isolation of individual cells of interest. Single cell isolation at blastocyst stages presents an additional challenge; it is a laborious process that requires fast, manual disaggregation of cells from embryos using a variety of strategies. Steps to label and sort subpopulations of cells have been taken [5,35,36,105,106]. However, besides the risk of introducing technical artifacts during sample processing, these approaches only allow the selection of a limited number of subpopulations of cells (thus far only 2–3). Alternatively, automatic cell sorting (for example using FACS) has been previously applied to mouse blastocyst samples, although it requires larger sample sizes than manual sorting [62]. Combinations of these approaches will undoubtedly yield higher resolution, temporal data and further insight on the events driving lineage specification in the embryo.

6. Concluding remarks

It is evident that developmental biology has been able to exploit recent technological advances — both on the bench and on the desktop. In this essay we have discussed some recent progress in harnessing these technologies to obtain high-resolution, single-cell level data from early mouse embryos. Current technology allows us to study global changes in gene expression upon experimental manipulation and to visualize virtually all cells and their interactions within the context of the live embryo. Given this level of resolution, the challenge ahead will be to bring these types of data together, and to develop formal mathematical models that will aid in experimental design and data interpretation to yield new insights into the genetic and cellular processes that shape the mammalian embryo.

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Figure 1. Early stages of mouse development

(A) Schematic of the preimplantation and early postimplantation stages of mouse embryonic development. Developmental time is indicated as embryonic days (E) from left to right below the corresponding embryonic stage. The main morphogenetic events are indicated in bold, italicized font. (B) Diagram representing the binary cell fate decisions taking place during preimplantation development. The tissues generated by each lineage later in development and the stem cells that can be derived from them are italicized. ICM: inner cell mass, TE: trophectoderm, EPI: epiblast, PrE: primitive endoderm, ExE: extraembryonic ectoderm, EPC: ectoplacental cone, TGCs: trophoblast giant cells, VE: visceral endoderm, ParE: parietal endoderm, TS: trophoblast stem, XEN: extraembryonic endoderm, ESCs: embryonic stem cells, EpiSCs: epiblast stem cells.

Figure 2.

(A) Alternative temporal sequences of events may lead to a single developmental outcome. These alternatives can only be revealed when studying events over a temporal scale. (B) Heterogeneities present in cell populations (for example, in terms of gene expression) become diluted when averaging a cohort of cells, but are revealed when analyzing individual cells. (C) Pipeline for transcriptional profiling of cells from embryos at single time points. Surface cells can be labeled and segregated, providing a certain degree of positional information. These approaches yield detailed information about gene expression but little data on other variables such as cell position. (D) Pipeline for live, *in situ* analyses. Individual cells can be tracked over time within the embryo to establish lineage trajectories, mapping retrospective and prospective histories. While these approaches yield lower resolution gene expression data, they capture other aspects of the developmental process such as cell movement or changes in cell shape.

Figure 3. Analysis pipelines for *in situ* approaches

(A) Live imaging using nuclear-localized reporters allows cell tracking and monitoring of changes in gene expression. Images at single time points can be segmented using image analysis software to identify and track individual cells throughout the embryo and over time. (B) Samples fixed at single time points can be labeled to determine the expression of multiple genes and/or proteins. Cell identities can be determined based on gene and/or protein expression levels as well as on spatial coordinates. Color coding in the top panels of (A) and (B), is as described in Figure 1 (TE: green, ICM: purple, PrE: blue, epiblast: red). Green nuclei in (A) represent nuclei reporter (generally GFP-based). Rainbow coloring in diagrams of z stacks in both (A) and (B) represent identification and labeling of single cells by image segmentation software.