A continuum of transcriptional identities visualized by combinatorial fluorescent in situ hybridization

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SUMMARY
Oligonucleotide-based fluorescent in situ hybridization (FISH) coupled with high-resolution high-sensitivity microscopy allows the visualization of single RNA molecules within fixed cells and tissues as distinct foci. We show here that combinatorial labeling of RNA molecules with several fluorescent dyes extends the number of genes that can be targeted simultaneously beyond the number of fluorophores used. This approach also inherently validates the identification of transcripts reducing false positive counts. We have used combinatorial FISH and image analysis to measure the transcript densities of six genes using three fluorophores. This has allowed us to visualize the endothelial maturation of lateral mesoderm in an in vitro ES differentiation assay from a single snapshot of molecular identities. Our observations show that, under these specific conditions, endothelial maturation follows a homogeneous course with a gradual increase in expression of Cdh5 and a concomitant loss of early transcription factors, arguing that maturation is governed in a generally deterministic manner. This methodology is limited by the number of fluorophores that can be used and by the available microscopic resolution, but currently available equipment should allow the visualization of transcripts from 10 or more genes simultaneously.

KEY WORDS: RNA visualization, Cell state, Differentiation, Fluorescent in situ hybridization, Single cell, Single molecule

INTRODUCTION
The primary unit of biological control is the cell. Measurement of cellular properties using bulk populations of cells is thus problematic unless a reasonable extent of homogeneity of identity or behavior exists within the population. As the output of development or cellular differentiation is the generation of cellular heterogeneity, the study of such processes requires simultaneous measurements at the single cell level of a sufficient number of parameters to describe the internal variance. As cell interactions are fundamental to developmental processes, the description of spatial arrangements is also desirable.

We therefore sought to develop methods for in situ multiparametric quantification of gene expression at the single cell level in large numbers of cells. Currently, existing methods for single cell multiparametric measurements can be broadly divided into cytometry and RT-PCR-based assays. Modern RT-PCR-based methods allow the simultaneous measurements of large numbers of parameters in intermediate cell numbers (~100); however, measurements rely on exponential amplification and expression estimates can vary by orders of magnitude from linear-based methods (Hayashi et al., 2008; Schwanhäusser et al., 2011) making data interpretation difficult.

Cytometry (e.g. fluorescence activated cell sorting; FACS) allows rapid multiparameter (up to 10) measurements on very large number of cells; recently, the combination of cytometry with mass spectrometry has allowed the large scale characterization of 31 antigens simultaneously (Bendall et al., 2011). Cytometry is, however, limited by the availability of suitable antibodies and its accuracy is difficult to assess.

In situ-based methods allow the localization of expressing cells, but have historically not provided quantitative expression measurements. Although it was shown in 1998 that oligonucleotide-based fluorescent in situ hybridization (FISH) combined with high resolution microscopy can be used to visualize individual mRNA molecules (Femino et al., 1998), this route to quantification has only been used to address very specific questions (e.g. Raj et al., 2006). More recently, Raj et al. (Raj et al., 2008) proposed the use of numerous short (20 nucleotide) probes labeled with single fluorophores for the identification of individual transcripts. This method has two primary benefits over previous methods: (1) the use of large numbers of probes targeted to individual transcript regions provides an amplification of the signal that is dependent on the sequence targeted; (2) a lower cost of probe synthesis allowing routine use. Such methods provide unambiguous quantification of transcripts but have been limited in mammalian cells to the measurement of three genes simultaneously (Itzkovitz and van Oudenaarden, 2011).

The use of multiple probes per transcript allows the encoding of transcript identities using a combination of fluorophores and it was demonstrated in 2002 that this can be used to detect the sites of transcription of more than 10 genes simultaneously (Levsky et al., 2002). This and subsequent work (Raj et al., 2006) demonstrated the stochastic nature of transcription, showing that active transcription cannot be used to describe cell state, as gene activation (a change in state) implies not continuous transcription but an increase in the probability of transcriptional events. Conceptually, the methods used by Levsky et al. (Levsky et al., 2002) can be used to detect individual transcripts; however, the use of a small number of probes per transcript (up to five used) do not provide an amplification of the signal in the absence of colocalization of large numbers of transcripts (observed at sites of transcription) resulting in a poor signal. Here, we show that the use of large numbers of short probes allows transcripts to be targeted by several fluorophores, allowing the simultaneous quantification of gene expression from more than six genes in cultures of mammalian cells. We also present a workflow for the development and verification of probe sets, FISH, microscopy and analyses of both raw image data and subsequent transcript counts.

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We have used these methods in combination with in vitro differentiation of mouse embryonic stem (ES) cells in order to test whether combinatorial FISH can be used to determine the manner in which cellular identities change. We have previously investigated the transition from primitive mesoderm to the endothelial lineage using both high-throughput and conventional methodologies (Kataoka et al., 2011; Sakurai et al., 2006; Yamashita et al., 2000). This has allowed us to identify genes that change during this transition; however, this has not provided a quantitative understanding of the manner in which gene expression changes during the process. Furthermore, the unknown heterogeneity of the bulk populations analyzed makes it impossible to determine co-expression or order of induction. Using combinatorial FISH, we show that it is possible to determine axes of both lineage and stage differentiation through a single snapshot of cell identities, and thus derive an order of events.

MATERIALS AND METHODS

Probe preparation and fluorescent in situ hybridization were carried out mostly as described previously (Raj et al., 2008), with some modifications in sample preparation, buffers and processing details. Reagents were obtained from Wako (Osaka, Japan) unless otherwise mentioned.

Probe design
As ~24 probes (each containing a single fluorophore) are required to detect transcripts reliably, a minimum of around 500 bases of a suitable target sequence is required for each fluorophore. In order to reduce differences in signal intensities owing to alternative splice forms, we first selected target sequences from exons common to most known splice forms. We then masked regions of those that have strong similarities to other transcript sequences using blast at http://www.ensembl.org/. The resulting sequences were used as input to the probe design tool at http://www.singlemoleculefish.com using default settings. We designed 48 or 72 probes per gene, allowing the use of either two or three fluorophores. We attempted to avoid non-coding regions (as these may contain more secondary structure), but given prior constraints (sequence similarity and common exons), we often used extensive non-coding regions as well as GC-rich regions. Probe sequences are in supplementary material Table S1.

Probe synthesis and labeling
Probes were ordered from Bios array Technologies (http://www.bioarraytech.com) with 3’ amino modifications in 96-well plates at a 5 nmol scale of synthesis. Dyes were purchased from Invitrogen (Alexa 488) or GE Healthcare (Cy3, Cy5) as NHS or 5-SDP esters. Probes were dissolved to a target concentration of 500 ng/μl on the basis of expected yield. For each gene, we created intercalated pools of probes containing 24 or 48 oligos (3 μl of each oligo). These pools were vacuum dried using a DNA110 Speedvac and dissolved in 20 μl 0.1 M carbonate buffer (pH 8.8) containing 140-200 ng of freshly dissolved fluorophore (NHS or 5-SDP esters). The mixtures were incubated overnight at 25°C with shaking. Unincorporated fluorophore was removed by gel filtration using NAP 5 Sephadex G25 columns (GE Healthcare) preceded by either ethanol precipitation or serial extraction with 1-butanol. Eluates were concentrated to a few μl by rotary evaporation and resuspended in 50 μl 5% acetonitrile (AcN) in 0.1 M triethyrammonium acetate (TEAA) (buffer A). Unlabeled and labeled oligonucleotides were separated using reverse-phase high-performance liquid chromatography (HPLC) using a gradient of 18 to 30% buffer B (65% AcN in 0.1 M TEAA) on a 0.5 ml C18 4 μm InertSill HPLC column (GL Sciences, Tokyo, Japan). Fractions containing labeled probes were pooled and subjected to one more round of gel-filtration and the concentration adjusted to 50 ng/μl.

Sample preparation
Combinatorial FISH was performed on cells grown on glass slides coated with E-Cadherin-IgG-Fc fusion protein (E-Cadherin-Fc: R&D Systems, 648-EC) (Nagaoka et al., 2006) (undifferentiated), or Collagen type IV (differentiation). E-Cadherin-Fc (10 μg/ml in PBS) and Collagen type IV (10 μg/ml in 0.1 M HCl) were adhered to poly-L-lysine (PLL) coated slides (Matsunami Glass, Osaka, Japan) for a period of 4 hours to overnight at 37°C. Slides were washed briefly with PBS before seeding cells. We used Grace Bio-Labs (http://www.gracebio.com) silicon gaskets (CW-8R-L0 CultureWell Gasket) to create eight wells with a diameter of 6 mm, into which 5000-20,000 cells were cultured for up to 2 days in 50 μl medium.

Embryonic stem (ES) cell maintenance and differentiation
CCE ES cells were maintained and differentiated as described previously (Yamashita et al., 2000). Mesodermal differentiation was initiated by plating 20,000 ES cells in CollIV-coated six-well plates (Becton Dickinson) with αMEM (Gibco, Life Technologies) containing 10% fetal calf serum. These cells were split on day four and 10,000-20,000 cells were transferred to CollIV-coated glass slides and cultured for up to 48 hours in SFO3 (Sanko Junyaku) medium supplemented with 30 ng/ml VEGF. EB3 ES cells were kindly provided by Hitoshi Niwa (Riken, Japan).

FISH
Cells grown on slides were briefly washed with PBS and fixed with 4% PFA at room temperature for 10 minutes. Following fixation, cells were washed three times (5 minutes each) in an excess of ice-cold PBS. Slides were then either dehydrated to 70% ethanol and stored at 4°C (for up to 2 weeks), or directly incubated with prehybridization buffer [PH: 10% formamide, 2×SSC, 0.1% Triton X-100, 0.02% BSA, 2 mM Ribonucleoside Vanadyl Complex (New England Biolabs, http://www.neb.com)] at 30°C. Stored slides were rehydrated prior to incubation with prehybridization buffer as above before FISH. Probe sets were combined to give a final concentration of 1-2 ng/μl per probe set. If used, unlabeled competitors were added at this stage and the mixtures concentrated by rotary evaporation to a few microlitres and resuspended directly in hybridization buffer (HB; PH + 10% dextran sulfate, usually 20-30 μl/well). Hybridization was carried out in a humid chamber (moistened by 10% formamide) at 30°C overnight. The hybridization solution was applied directly to the wells formed by the silicon gasket and left uncovered. The following day, slides were washed twice with 10% formamide, 2×SSC, 0.1% Triton X-100 at 30°C for 30 minutes each. Slides were then transferred to PBS, stained with DAPI (0.5 μg/ml in PBS for 1 minute), briefly washed with PBS, dehydrated and mounted in Prolong Gold or freshly prepared Prolong antifade (Invitrogen) mounting medium. Slides were viewed on the following day, and, if sealed (with nail polish) and stored dry at ~20°C, could be kept for up to 6 months without major degradation in the signal.

Probe competition
FISH was carried out as described, but including an excess (5 ng/μl) per probe of unlabelled oligonucleotides in the hybridization mixture.

Imaging
Slides were imaged using a DeltaVission Core (Applied Precision, http://www.appliedprecision.com/) system equipped with a 60× 1.42 NA lens (Olympus), using standard filters for DAPI, FITC, TRITC and Cy5 illuminated either by a mercury or xenon light source. Image stacks were created using 200 nm step sizes with a 1024×1024 camera giving final voxel dimensions of 107×107×200 nm (xyz). The resulting image stacks were deconvolved using the DeltaVision deconvolution program (decon3d) using 10 iterations with default settings.

Image analysis
Image stacks were viewed and analyzed by functions implemented in a custom application (dvreader) written in C++ using the Qt4 graphical user interface toolkit. All source has been released under the GPL (http://www.gnu.org/copyleft/gpl.html) and is available at http://www.gitorious.org/dvreader.

We used a thresholded three-dimensional watershed algorithm to initially define local maxima (blobs); blobs were initiated when intensity values exceeded a specified minimum (optionally after applying a local background subtraction). Only blobs with peak values above a user-specified threshold were included in further analyses. Transcript identities were assigned by identifying blob peaks from additional fluorescent
channels with a peak-peak distance of less than 1-2 voxels after correcting for apo-chromatic- and emission filter-induced shifts. The resulting sets of overlapping blobs (blob sets) were filtered using parameters describing the constituent blobs (e.g. mean, max voxel intensity, blob volume, etc.). Allowable ranges for the parameters were set manually for each individual fluorophore and transcript id, as signals using different fluorophores and probe-sets have different expected properties (e.g. larger probe numbers give stronger signals). In the experiments described here, we have simply dropped blob sets that do not conform appropriately, but one can envision an iterative classification and filtering approach.

Nuclear segmentation
Nuclei were segmented by a simple tracing algorithm that is initiated upon encountering a pixel above a given threshold. Once such a pixel is encountered the algorithm searches the immediate space around that pixel for a maximal neighboring pixel above the threshold and iterates until it encompasses a region. The resulting perimeters were filtered by length followed by manual inspection and editing where necessary.

Cell segmentation
Blob sets were assigned to nuclei using a nearest-neighbor method. Clusters of blob sets were seeded from unassigned blob sets and expanded iteratively by identifying the blob set lying closest to any member of the cluster. The expansion was stopped, either when within a minimum distance of a nucleus, or when incorporating a member of an already assigned cluster. The resulting cell perimeters were manually checked and amended when necessary using functions built into the dvreader application. Although blob sets were mapped in three dimensions, both nuclear segmentation and manual correction of cell boundaries was performed in two dimensions, resulting in inevitable overlaps. Transcripts falling within such overlapping regions were omitted from further analyses.

Data were analyzed using both R functions and a custom-written application for summarizing n-dimensional relationships in two-dimensional space. The source code to this can be obtained from the sod/directory of the dvreader source.

RESULTS
Combinatorial FISH tested in ES cells
To test hybridization conditions for combinatorial detection of individual transcripts, we first designed probes against *Fgf4* and *Nanog*. These genes are both expressed at high levels in undifferentiated ES cells and their expression is lost upon differentiation. Forty-eight 20-nucleotide probes were designed against both genes. The probes were combined into pools of odd- and even-numbered probes, and each pool labeled separately with either Cy3 or Cy5. Transcripts from both genes could thus be probed either with single colors (*Fgf4*, Cy3; *Nanog*, Cy5; 48 probes each) or with both fluorophores in combination (24 probes each). ES cells hybridized with singly labeled probe sets against both genes contained large numbers of bright singly fluorescent foci (Fig. 1B, left), demonstrating a general lack of colocalization of transcripts from the two genes. By contrast, cells hybridized with probe sets targeted against a single gene (*Fgf4* or *Nanog*), but labeled with Cy3 and Cy5, contained, almost exclusively, fluorescent foci labeled by both fluorophores (Fig. 1B, middle and right).

The almost complete overlap between channels suggested that the vast majority of signals are due to the presence of the targeted transcript. However, the presence of a small number of singly
labeled foci of similar size and intensity to the major signal indicates the need for extensive validation of probe sets if used to detect genes expressed at very low levels.

**Expanding the probe set repertoire**

We next designed probes against a number of angiogenic and hematopoietic genes (Etv2, Fli1, Cdh5, Cdh5n, Gata2, Gata1, Tal1 and Runx1) to facilitate the investigation of the differentiation of blood and endothelial lineages. Each probe set contained either 48 or 72 individual probes to allow single, double and triple labeling of individual transcripts. Probes were combined into either odd and even, or into sets containing every third position for probe sets containing 48 or 72 probes, respectively. Individual pools of probes were labeled with Alexa 488 (a488), Cy3 or Cy5 and tested on both ES cells (which should not express any of the genes) and on ES-derived ECs.

Approximately half of the probe sets tested gave rise to expected signals; however, the remaining probe sets caused massive background signals when used in FISH (Fig. 2). In order to identify background-producing probes we attempted to compete out the background using unlabeled probes. In most cases, background signals could be removed by the inclusion of an excess of one or a small number of unlabeled probes. In a few cases, we were unable to pinpoint all problematic probes and in one case (Gata1) we have as yet been unable to reduce the background to satisfactory levels.

We observed a range of different categories of background ranging from smooth cytoplasmic staining, globular nuclear and speckled to one or two extremely bright diffraction limited foci per nucleus (Fig. 2). Most of these probes cause similar signals in both ES and differentiated cells (mesoderm and mesoderm derivatives), suggesting that they interact with parts of the basic cellular apparatus.

We then used competitors to allow FISH with complete probe sets on cultures containing ECs to evaluate the performance of combinatorial FISH. FISH using singly labeled probe sets resulted in almost exclusively non-overlapping fluorescent dots (Fig. 3A). By contrast, using three fluorophores to target four genes with combinatorial labeling (Tal1 a488/Cy5, Gata2 a488/Cy3, Etv2 Cy3/Cy5 and Fli1 a488/Cy3/Cy5) resulted almost exclusively in fluorescent foci that overlapped between channels (Fig. 3B). This lack of non-overlapping signals prompted us to use a combination of both singly and combinatorially labeled probe sets. This resulted in the appearance of six classes of fluorescent foci with the expected combinations (Fig. 3C; Fig. 4A). As the resulting colors reminded us of those in a bowl of candy, we propose the term candyFISH to refer to this type of analysis.

To increase the number of usable fluorophores, we also used mega-stokes dyes (Dyomics, http://www.dyomics.com/) that can be distinguished from more standard dyes by using excitation at shorter wavelengths (owing to their larger Stokes shift). These dyes are not very bright, and are not suitable for use in combinatorial detection; however, they can be used in singly labeled probe sets allowing us to expand the number of genes targeted simultaneously (Fig. 3D, seven genes: Gata2 a488, Tal1 Cy3/Cy5, Runx1 Cy3, Etv2 Cy5, Kdr a488/Cy3, Pdgfra a488/Cy5 and Fli1 Dy520). This allows the extension of the number of distinct type of foci that can be distinguished; but the analysis of the resulting images is complicated by the appearance of some degree of fluorescent resonant energy transfer (FRET) at combinatorially labeled transcripts. The use of more tightly spaced conventional fluorophores (e.g. Levsky et al., 2002) should more easily extend the number of usable dyes, but we have been limited by the filter sets available to us.

**Converting image to expression data**

In order to obtain image data from more (complete) cells, we collected image data from overlapping three-dimensional image stacks giving total dimensions of 400-500 µm in the xy plane. We then used a thresholded watershed algorithm to detect three-dimensional local peaks (blobs) for each fluorescent channel and assigned transcript identity on the basis of overlap between fluorescent channels.

Although it is conceptually easy to enumerate transcripts, it is not clear how to assign transcripts to individual cells. This is especially true in cultures of differentiating cells in which...
assumptions regarding cell size and shape that have been used previously (Allalou and Wählby, 2009) do not apply. Although it may be possible to delineate cell boundaries using membrane markers, in practice these often do not work well in mesodermal cells and doing so would require an additional fluorophore channel. Instead, we used the observation that transcript density tends to increase with proximity to the nucleus by implementing a nearest-neighbor method that extends clusters of blobs to their nearest neighbor until merging with a nucleus (Fig. 4B, top left). Hence, we first defined nuclear borders and then assigned transcripts to individual nuclei.

The assigned transcripts were used to create two-dimensional cell borders to assess the accuracy of transcript assignment. Although the method produced a plausible transcript assignment (Fig. 4B, top right), an inspection of transcript composition indicated that errors do occur (Fig. 4B, bottom left), and we manually edited borders where necessary. The resulting cell outlines are restricted to two dimensions (though transcripts are mapped in three dimension) and this limits us to analyzing monolayers of cells. However, these observations suggest the use of transcript composition as a means to define cellular units (this being implicitly used in manual editing), though this is complicated by intracellular transcript localization and is by no means straightforward to implement.

Tracking the mesoderm to endothelial transition
To demonstrate the utility of this technology, we addressed a set of questions related to the differentiation of mesoderm to ECs. The phenotype of the Env2-null mutant indicates that ECs are derived from Env2-expressing mesoderm (Lee et al., 2008) and it has been shown that Env2 can drive the expression of endothelial genes (De Val et al., 2008; Hayashi et al., 2012). Env2 has also been shown to be a direct inducer of Tal1, Fli1 and Gata2 (Kataoka et al., 2011), which are thought to stabilize the hematopoietic state (De Val and Black, 2009; Pimanda et al., 2007). Hence, the course of gene induction caused by Env2 during endothelial differentiation remains unclear.

We derived a mixture of endothelial and non-endothelial cells by differentiating ES cells on collagen type IV (ColIV) as described previously (Yamashita et al., 2000). This differentiation procedure generally results in the appearance of about 50% ECs (as indicated by Cdh5 expression) and 50% smooth muscle actin (Acta2)-expressing cells. These cells were fixed and hybridized with probes against six genes that mark the endothelial and hemangiogenic lineages (Tal1, Runx1, Fli1, Gata2, Cdh5 and Env2). We collected data from three image sets (supplementary material Fig. S1) and quantified transcript numbers (supplementary material Table S2).

Within the 124 cells assayed, total transcript counts varied from 0 to 831 (Fig. 5A). The total number of transcripts varied both with lineage and cell size, with large transcript counts only being observed in large cells. Slightly more than two-thirds of the cells expressed substantial numbers of endothelial transcripts; those that did showed co-expression of all genes, but with low levels of Runx1 and variable levels of Env2. Somewhat surprisingly, a proportion of non-endothelial cells contained low levels of Runx1. No cells contained Runx1 transcripts at levels comparable with those observed in embryonic cells (data not shown), suggesting that Runx1 expression in these cells is below functional levels.

Although we observed co-expression of Gata2, Fli1 and Tal1 with Cdh5, the expression levels within cells did not correlate with Cdh5 expression but rather showed anti-correlation, with high levels of Cdh5 being associated with lower levels of these transcription factors (Fig. 5B), suggesting that the endothelial state is stabilized by other factors or mechanisms. This anti-correlation was particularly strong for Env2, consistent with its role in the initiation of lateral mesoderm differentiation. By contrast, we observed a strong correlation in expression levels between Gata2, Fli1 and Tal1.
With the exception of Etv2, and to some extent Gata2, gene expression levels formed a continuum of intensities between the gene maximum and minimum. By contrast, the maximum level of Etv2 expression was separated from lower levels, with only one or two cells expressing intermediate levels (Fig. 5B, Fig. 6C, D). To quantify this, we calculated the distribution of transcript numbers expressed as a proportion of the maximal count of each gene (Fig. 5C). This confirms that the distribution of Etv2 expression levels is distinct from that of the other genes.

We used an error minimizing algorithm to display the relationships between cells observed in this experiment (Fig. 6A). In order to minimize the impact of cell size, we used transcript densities estimated by dividing transcript counts by cell areas. Given that Etv2 is required for the appearance of endothelial or hematopoietic cells, we can assume that cells expressing markers for these lineages have expressed Etv2; similarly, we can to some extent use Etv2 expression as an indicator of differentiation stage, as our observations suggest that it is generally expressed as a pulse. This analysis indicates that the population of cells containing appreciable quantities of transcripts can be divided into three or four main components: (1) a single cell expressing high level of Etv2; (2) a number of cells expressing low levels of Runx1; (3) a main body of cells lying on an apparent axis of endothelial maturation; and (4) a handful of outlier cells characterized by high levels of Tal1 (Fig. 6A).

It follows from this that we can divide the heterogeneity within the population into chronological (or extent of differentiation) heterogeneity, with the majority of the population proceeding along an axis of endothelial differentiation starting from Etv2 high/Tal1, Fli1, Gata2 (TF) low/Cdh5 low and proceeding to an intermediate Etv2 low/TF high/Cdh5 intermediate and finally to an Etv2/TF low/Cdh5 high identity.

To provide a quantitative view of the change in expression along this axis, we defined a vector of differentiation running from cell 20 (Etv2 high) to cell 45 (Cdh5 high) within the reduced dimensionality space (Fig. 6A). Cells were mapped onto this vector and filtered by their distance from it to include the main endothelial population (within parallel lines in Fig. 6A). Means and standard deviations of a sliding window (width, 0.2; slide increments, 0.005; where 1 is the full length of the vector) were then calculated (Fig. 6C). Expression of Cdh5 increased concomitantly with a decrease in Etv2, Tal1, Fli1 and Gata2. To visualize the variance in expression along the vector, we plotted individual transcript densities versus position (Fig. 6D). This shows a homogeneous linear increase in Cdh5 expression along the vector, with the decrease in expression of Fli1 and Tal1 being characterized by a larger variance.

In addition to the main population, there are at least two cells that seem to mature (i.e. low Etv2, but high Tal1 and Fli1) without activation of Cdh5. However, as these cells are rare, it is difficult to determine their relationship to the main endothelial population. However, we can refer back to the original image data sets to confirm that these data points are not due to measurement artefacts. Indeed, these cells not only show a difference in their transcriptional identity, but also in their shape: having a less flattened morphology as an indicator of differentiation stage, as our observations suggest that it is generally expressed as a pulse. This analysis indicates that the population of cells containing appreciable quantities of transcripts can be divided into three or four main components: (1) a single cell expressing high level of Etv2; (2) a number of cells expressing low levels of Runx1; (3) a main body of cells lying on an apparent axis of endothelial maturation; and (4) a handful of outlier cells characterized by high levels of Tal1 (Fig. 6A).

In the above analysis, we have inferred a chronological heterogeneity on the basis of Etv2 and Cdh5 expression. In order to confirm this, we carried out FISH at 6, 15, 24 and 48 hours after replating in VEGF containing SF03 medium. In order to exclude the possibility that the observed anti-correlation between Fli1 and Cdh5 was affected by misclassification of Fli1 transcripts due to high concentrations of Cdh5 transcripts (which share one
fluorophore), we used non-combinatorial FISH targeted against Etv2, Fli1 and Cdh5 (Fig. 6E).

Six hours after VEGF exposure, we observed a small number of cells containing a range of Etv2 transcript numbers, some of which contained very low levels of Fli1 or Cdh5. At 16 hours, Etv2-positive cells were common, and most of those also contained Fli1 and Cdh5 transcripts. At 24 hours, we observed a similar situation to that described above: most cells contained a mixture of Fli1, Etv2 and Cdh5; there was a small number of Etv2 high cells; and Cdh5 high cells contained lower levels of Fli1. At 48 hours, most cells in the endothelial lineage contained very high densities of Cdh5 transcripts but much lower levels of Fli1. Very few Etv2 transcripts were observed at this time.

**DISCUSSION**

Here, we demonstrate the feasibility of using combinatorial FISH to simultaneously measure RNA abundance from more than six genes in single cells. We have used this to analyze how cellular identities change during the differentiation of primitive mesoderm to an endothelial identity. This is a well-characterized process that has been studied in detail, resulting in the identification of a small number of essential genes [Vegfa (Carmeliet et al., 1996), Kdr (Shalaby et al., 1995) and Etv2 (Kataoka et al., 2011; Lee et al., 2008)] and a large number of genes that are activated and or repressed during the process. However, our previous attempts to describe the process based on microarray data of sorted populations were frustrated by the inability to determine whether observed co-induction of genes was occurring within individual cells. To test the possibility of using combinatorial FISH to clarify this situation, we concentrated on six genes known to be involved in the development of the endothelial and hematopoietic lineages. Etv2 is absolutely required for the appearance of both lineages (Lee et al., 2008), though its expression both in embryos and in vitro seems to be limited to a brief window associated with the transition from a primitive to lateral mesoderm identity. Tal1, Fli1 and Gata2 appear to be crucial targets of Etv2, and their
Fig. 6. Cell identities. (A) Nodes representing individual cells were arranged in a two-dimensional space using a heuristic that attempts to maintain cell-cell relationships based on cell expression profiles. Each pie-chart represents a single cell. Distances between charts indicate euclidean distances between cells calculated using transcript densities; scale bar indicates distance in units of transcripts per 1000 μm², as in Fig. 5B. Pie segment areas are related to the total transcript density (transcripts/unit area) and the segment sizes are proportional to the individual gene transcript densities. Five main cell identities can be discerned and are indicated in the figure as: (i) cells expressing very low or no transcripts, (ii) cells expressing primarily low levels of Runx1, (iii) a single Etv2 high cell, (iv) a small number of cells containing high levels of Tal1 but low levels of Cdh5 and (v) a main field of cells expressing variable levels of all the genes assayed. The primary axis of differentiation within this population (arrow) of cells seems to extend from the lower-left to the upper-right corner, with a graded loss of Etv2 being accompanied first by an increase in Cdh5 and endothelial transcription factor expression, followed by a further increase in Cdh5 and loss of Gata2, Tal1 and Fli1 expression. Arrow connecting cells 20 and 45, and lines parallel to it, indicate vector and filtering used in C. (B) Images of cells taking extreme positions in A. Numbers indicate cell IDs as shown in A. Alexa 488, blue; Cy3, green; Cy5, red. (C) Change in expression along the inferred route of endothelial maturation. Cells were projected onto a vector running between cells 20 and 45. Mean transcript densities for cells lying between the parallel lines in A were calculated for a sliding window running along the vector. Error bars indicate standard errors; numbers along the x-axis indicate the number of cells in each window (each cell is represented in five window positions). (D) Plot of individual transcript densities versus inferred differentiation for Cdh5, Etv2, Tal1 and Fli1 (each vertical stack of points represent a single cell). Transcript densities are given as transcripts per 1000 μm². (E) ES cells were differentiated on collagen in the presence of serum and then replated in serum-free medium with VEGF for the indicated times. FISH was performed in a non-combinatorial manner against Cdh5, Etv2 and Fli1.
expression overlaps with that of En2 in early embryos (Katoaka et al., 2011). Cdh5 encodes a cell-adhesion molecule that is crucial for endothelial function though dispensable for endothelial differentiation (Gory-Fauré et al., 1999). Runx1 is crucial for definitive hematopoiesis and is expressed in lateral mesoderm (Sakurai et al., 2006). Expression in endothelial or endothelial descendants seems to be necessary for the appearance of hematopoietic stem cells (Chen et al., 2009). However, data from in vitro differentiation suggests that Runx1 is not directly regulated by En2 (Katoaka et al., 2011).

Hence, we would expect that En2 activation is followed by expression of Tal1, Fli1 and Gata2, followed by Cdh5, with a gradual reduction of En2 coinciding with an increase in Cdh5. Indeed, this is almost exactly what we observe. Furthermore, as we can observe a continuum (albeit interrupted) of cell identities from a presumably primitive (En2 high, others low) to a more differentiated state (Cdh5 high), we can infer this route of differentiation from a single timepoint.

Since expression of Cdh5 was observed in the least differentiated cells (i.e. high En2) that only contained low levels of Fli1, Tal1 and Gata2, we can surmise that Cdh5 is also a direct target of En2; although as its expression is increased during the process, control must pass to other factors. Under these conditions Tal1, Fli1 and Gata2 seem to behave in a similar manner to En2 in that their expression seems to be shortlived and lost upon endothelial maturation. Somewhat pleasingly, we see a good correlation in expression between Tal1, Fli1 and Gata2. These three genes are thought to form a positively reinforcing triad motif that stabilizes their expression in hematopoietic precursors (Pimanda et al., 2007). This is consistent with our observations; however, as their expression is lost during endothelial maturation it seems that the correlation may be due not to internal feedback within the loop but due to common external regulation.

Although we observed a gradual reduction in levels of Fli1, Tal1 and Gata2 with an increase in Cdh5 expression (Fig. 5C; Fig. 6A,C,D), the reduction in En2 levels drops rapidly, suggesting that the high En2 state is unstable and rapidly resolves itself to an En2 low state. Hence, although these transcription factors (En2, Tal1, Fli1 and Gata2) are all expressed as a pulse during the differentiation process, the manner in which expression is lost differ, suggesting different modes of regulation.

In contrast to previous expectation (Levsky and Singer, 2003), our data suggest that under these conditions, endothelial differentiation is remarkably homogeneous. All cells expressing Cdh5 (marking this lineage) also contain appreciable levels of all the other factors, and their transcript densities seem to be related to the extent of differentiation. Outside of this main population we also observe around 20 cells that contain no, or very low levels of, these transcripts; and we also observe two or three cells with high levels of Tal1 and Fli1. This low level of heterogeneity is probably, partly to at least, caused by the use of defined conditions (no serum) and a two-dimensional culture system that can provide cells with a homogeneous environment (Nishikawa et al., 2007).

These data suggest that the process of endothelial maturation follows a consistent course that does not appear to be under much stochastic influence; variance within the endothelial population appears to be caused by differences in the timing of lineage entry. By contrast, lineage entry appears to be stochastic, suggesting that the processes of lineage choice and maturation are governed by different regulatory mechanisms. However, this cannot be concluded from our present data as we have little information regarding the heterogeneity of the initiating population.

Taken together, the data presented here show not only that combinatorial FISH can be used to quantitate transcription from multiple genes at the single cell level, but also that this can be sufficient to track the change of cellular identity during differentiation processes. However, we have only been able to implement these methods in monolayer cultures owing to the difficulty of accurately segmenting cells in three dimensions and the increased autofluorescence associated with thicker tissues. Although we have been able to visualize individual transcripts in whole-mount embryos (mouse E7.5-8.0) using longer wavelength emitting dyes (Cy3/Cy5), the signal-to-noise ratio has not been sufficient to allow for combinatorial detection. This problem can be overcome by disassociating cells followed by immobilization on slides for microscopy. Though this will result in the loss of spatial information, this can to some extent be abrogated by performing FISH on cells sorted by fluorescence cytometry. Recent developments (Cella Zanacchi et al., 2011) in light-sheet microscopy (Greger et al., 2007) may be sufficient to make the methods outlined here applicable to thicker tissues. Such developments should also abrogate the problem of 3-D cell segmentation due to the associated increase in resolution in the Z-axis. Our approach is limited by the number of fluorophores and the imaging resolution; however, Lubeck and Cai (Lubeck and Cai, 2012) have recently demonstrated the detection of transcripts from 22 genes in yeast using switchable fluorophores and super-resolution microscopy. Although the imaging used by Lubeck and Cai (Lubeck and Cai, 2012) is currently only suitable for very thin (~100 nm) samples due to relying on near-field microscopy we believe this will change in the future and that the number of transcripts that can be targeted will drastically increase.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References
Supplementary Images
Maximum intensity projections of the three image sets (panels 1-3) used to derive transcript counts summarised in main figures 5 and 6, and in supplementary table 2. Image set ids are denoted in supplementary table 2.

CCE ES cells were differentiated for four days in aMEM 10% serum on Collagen type IV (Col IV). At day four, 20,000 cells were re-plated in a 6mm well on a Col IV coated glass slide and re-cultured for a further 24 hours in serum free medium (SFO3) and VEGF (30 ng/ml). The cells were then hybridised with probes against Cdh5 (Cy3), Gata2 (a488*, Cy3), Etv2 (Cy5), Tal1 (a488, Cy5), Runx1 (Cy3, Cy5) and Fli1 (a488, Cy3, Cy5) as described in the main text. Colours are: DAPI white, a488 blue, Cy3 green, Cy5 red, resulting in the following labelling: Cdh5 green, Gata2 cyan, Etv2 red, Tal1 magenta, Runx1 orange (~ish) and Fli1 whitish. Note that single coloured labelled transcripts give much stronger signals (eg, Cdh5 and Etv2) which tend to obscure the lower intensity combinatorially labelled transcripts when viewed by eye. This is especially true for Gata2 since the number of Gata2 transcripts is generally low. The images were derived from overlapping z-stacks merged using the dvreader application (http://www.gitorious.org/dvreader). Pixel dimensions are 107 x 107 nm. All three images were viewed with the same viewing parameters (i.e. brightness / contrast). *a488 = Alexa fluor 488. Original data and non-compressed tiff files of the projections are available from the authors upon request.