ESC-Track: a computer workflow for 4D segmentation, tracking, lineage tracing and dynamic context analysis of ESCs.

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Abstract

Embryonic stem cells (ESC) can be established as permanent cell lines, and their potential to differentiate into adult tissues has led to their widespread use for studying the mechanisms and dynamics of stem cell differentiation and exploring strategies for tissue repair. Imaging of live ESCs during development is now feasible due to advances in optical imaging and engineering of genetically encoded fluorescent reporters, with the major limitation being the low spatio-temporal resolution of long-term 3D imaging required for generational and neighbouring reconstructions. We present the ESC-Track (ESC-T) workflow, which includes an automated cell and nuclear segmentation and tracking tool for 4D (3D+t) confocal image datasets, and a manual editing tool for visual inspection and error correction. ESC-T automatically identifies cell divisions and membrane contacts for lineage tree and neighbourhood reconstruction and computes quantitative features from individual cell entities, enabling analysis of fluorescence signal dynamics and localization of cell morphology and motion. ESC-T is a powerful tool allowing the evaluation of genealogical and microenvironmental cues that maintain ESC fitness. Here we present the use of ESC-T to examine Myc intensity fluctuations in the context of mouse ESC (mESC) lineage and neighbourhood relationships.

Introduction

Stem cells provide essential functions during embryonic development and tissue regeneration. Mouse ESC (mESCs) are derived from pluripotent cells of the early mouse embryo and can be maintained as stable cell lines with a high self-renewal capacity. They provide a versatile in vitro model for understanding differentiation of human tissues and their study has led to major advances in cell and developmental biology (reviewed in (1, 2)). A key challenge in the field is to understand the mechanisms involved in guiding the fate of stem cells (3–5), which has broad applications in biomedicine; from elucidating the causes of cancer to the use of stem cells in regenerative medicine. Thus, the biological properties of ESCs are a matter of great scientific, commercial, and public interest.

Advances in optical imaging has led to the emergence of powerful live imaging tools with individual cell resolution in three-dimensional (3D) space and in time (3D+t or 4D) (6, 7). Moreover, new generation of fluorescent proteins and dyes allows biochemical characterization of signaling pathways in intact living cells (8). Fluorescent protein tagging enables tracking the position of any given cell over time, which is easily achieved when the population of tagged cells is distributed among non-expressing cells by virtue of lineage or in a mosaic experimental situation, but it becomes challenging when a fluorescent protein label is widely expressed (9). The ability to track and analyse live cells in time lapse 4D microscopy images is a matter of intense research (10, 11) since visual inspection and analysis are insufficient to extract meaningful insights, being automated tracking and quantitative analysis of cells an absolute requirement. This is such a challenging task that several competitions have been carried out in

order to evaluate cell segmentation and tracking algorithms (12, 13). Computational tools are essential for extracting quantitative measurements from stem cell populations growing in 3D physiological conditions, and to translate them into biological knowledge allowing the study of a range of cell behaviours such as; motility, cell division, death, phagocytosis, etc. Most of the methods have been applied to Drosophila (14–18) and Zebrafish (19–21) embryogenesis, or plant morphogenesis (22) studies. Of special relevance in the field of stem cell biology is the ability to integrate the cell behaviour analysis with information about lineage (parent-progeny) and context (neighbourhood) cellular relationships (9, 11). In the last decade, several generic processing and tracking packages, such as Icy (23), Cell Profiler (24), tTt, qTfy (25), or the Fiji plugin TrackMate (26, 27) have been reported. Some complex methods for particle (28), nuclear (29, 16) or cellular (22, 20, 21, 17, 18) segmentation including; MARS (22), ACME (21), EDGE-4D (17), RACE (18) and for tracking, which include STARRYNITE (29), U-TRACK (28), ALT (22), EDGE-4D (17), and TGMM (16) have been developed for specific applications.

We present herein a computational workflow that allows the automated segmentation and tracking of individual mESCs from live cell 4D confocal image datasets based on the combination of membrane and non-homogeneous nuclear signals allowing lineage and neighbourhood reconstruction. The workflow enables the extraction of parameters relative to fluorescence signal localization and dynamics, to cellular morphological characteristics and to motion related aspects from individual cells in the context of lineage and neighbourhood relationships. ESC-T was used to study Myc dynamics in mESC cultures, and proved to be a very valuable computational tool in stem cell research, as it allowed the evaluation of genealogical and microenvironmental cues during mouse ESC culture in an unprecedented manner.

Materials and Methods

Image processing and analysis

- **Automatic cell and nuclei segmentation and cell tracking.**

The proposed pipeline (Fig. 1) uses images obtained from ESCs expressing tdTomato and GFP-MYC signals as described in Supplementary Materials and Methods. Pre-processing step consist on median filtering combining both nuclei and membrane signals (mycGFP median minus tdTomato median) (Fig. 1, steps 1-4) and is followed by 2D watershed segmentation algorithm, rendering 2D sets (cell portions) (Fig. 1, step 5). Spatiotemporal (3D+t) association rules based on sets overlap are applied to connect sets in 3D space and time for automatic segmentation and tracking through the following pipeline:

a) 3D cells are defined by 3D connection of 2D sets based on direct connectivity (Fig. 1, step 6).
b) As non-homogenous nuclear staining prevents from direct nuclear segmentation, a nuclei prediction image is generated by applying morphological erosion on the cellular segmentation mask and then combining it with a filtered version of the nuclear GFP-MYC channel (median filter with 2D kernel of size 5). Seeds are automatically detected inside 3D cells and develop following a 3D region growing algorithm for nuclei segmentation based on the nuclei prediction (Fig. 1, step 7).
c) Then, a hierarchical strategy uses a loop over 3D nuclei overlap (ratio of the intersected area to the area of the current object) to connect nuclei in timepoint ***t*** with unconnected nuclei in time point ***t-1*** or ***t-2*** (Fig. 1, step 8). Overlapping criteria for

connecting current and candidate nuclei is initially 0.7. For remaining unconnected nuclei, this connectivity criteria is reduced by 0.1 in a loop until achieving 0.1.

d) Outliers regarding nuclei sizes (number of voxels) below a threshold which is manually set by the user (Typically 100 a.u.) are removed (Fig. 1, step 9).

e) Nuclei remaining unconnected in time are re-analyzed for further classification (Fig. 1, step 10) as either; pieces of preexisting cells, new cells arriving to the imaging volume, or newborn cells from cell division.

In order to identify non-highlighted cell divisions during automatic tracking, additional criteria are considered in spatiotemporal association rules including biological knowledge about the minimum temporal distance between mitosis and nuclei shape before mitosis (30–32).

If two nuclei in ***t*** have been connected based on temporal overlap criteria to the same nucleus in ***t-1***, they are considered as daughter candidates resulting from cell division. In these cases, further criteria are determined:

a) Volume overlap ≥0.1 (relative to each daughter) for the two daughter candidates.

b) Volumes of candidate daughter cells with respect to the mother cell ≤0.5.

c) Last division of mother cell earlier than 4 hours.

d) Nuclei shape index before cell division ≤0.9 (index ranging from 0 to 1 , being 0 high and 1 low smooth surface and roundness).

e) Division maintained in time. Daughters appear as segmented cells >70 minutes after they are born.

If cell division criteria are fulfilled, candidates are classified as newborn daughter cells, receiving a new identification number with information on their progenitor. If volume overlap ≥0.1 for the two daughter candidates but any of the other three conditions is not fulfilled, then both cells are considered as different sets of the same pre-existing cell and therefore merged and named with the same ID. If volume overlap <0.1 between a cell at *t0* and any other cell at *t-1* or *t-2*, it is classified as a new cell arriving to culture. (Fig. 1, step 10). After classification, cell segmentation is updated with tracking feedback. IDs are structured as follows: the integer part represents the family, and the decimals identify daughters, with a decimal position being added at each generation (Fig. 2a).

- **Manual edition and feature extraction.**

A visualization and editing interface was implemented to allow the user for error correction in segmentation, tracking or cell division detection, as well as for identification and manual annotation of additional apoptotic processes (Fig. 1, step 11). Features are automatically extracted from the segmented and tracked nuclei at each time-point. Features include cell and nuclei morphometric parameters and GFP-MYC intensity distributions among others, as detailed in Supplementary Table S1. Tables are generated for each time lapse experiment in which each row represent one cell at one particular time-point. The number of cells analysed varied from 14 to 145 per mESC colony culture.

- **Lineage tracing and context analysis**

Cell identification with ID allows connecting dividing cells with their progeny, since each daughter cell ID carries information of its predecessors (Fig. 2a). In addition, for every cell and

time-point, cell neighbourhoods are identified as cells whose membranes are in direct contact with the cell of interest.

A specifically designed tool allows representation of cell lineages as trees for visualizing any of the features extracted from each cell and time-point within the lineage tree (Fig. 2) allowing:

a) Generating CSV files with selected cell families and calculation of parameters such as times to birth, division and death.
b) Visualizing cell IDs and extracted features from the data table as lineage trees (Fig. 2a); examples represent mean nuclear GFP-MYC intensities (Fig. 2b), GFP-MYC maximum intensity of neighbour cells (Fig. 2c), and nuclear sizes (Fig. 2d). Mean and maximum GFP-MYC intensities of neighbour cells are especially relevant for cell competition studies.
c) Filtering criteria for lineage tree visualization, such as, temporal resolution, image quality, cell family identity and number of families to be displayed.

Additionally, normalization of mean Myc values compensating for intensity loss along time is applied as described in Supplementary Materials and Methods. Once the tracking is completed for an entire image dataset, aggregated measurements that use the whole cell track information are computed for complete cell lifetimes; allowing the visualization of the trajectories of single cells together with representation of any parameter included in the feature table (Supplementary Table S1). Three outputs can be produced:

a) Full trajectories and colour-coded parameter values (Fig. 2e), including position at each time-point and cell division.

b) 2D videos of the Z-projection of the tracked cell(s) with colour-coded parameter values.

c) Combination of the previous two outputs in a single video where each frame shows the position of the cells and their feature value coded with colours, together with the trajectories followed from the beginning to the current timepoint (Fig. 2f and Video_S1).

Results and Discussion

The work is focused in studying Myc dynamics in the context of cell competition from live 4D confocal images of mESC expressing tdTomato and GFP-MYC. This study required quantification of fluorescence intensity parameters across neighbour relationships and the integration of this neighbouring analysis within the lineage trees based on prior automatic cell tracking from 4D image datasets. One of the main challenges when acquiring live cell 4D images is optimizing spatial and temporal image resolution while minimizing photo bleaching and photo-toxicity, and allowing enough time to capture cell processes (division, apoptosis and cell-cell interactions). Live 4D confocal imaging of Myc in mESCs required under sampling in Z-dimension, which diminished resolving power introducing a clear limitation in axial resolution affecting image processing steps (Fig. 3). The difficulty of obtaining accurate tracking results due to limitations on image resolution has been previously pointed out on several publications (22, 20). Added to difficulties of automatic tracking of images with strong limitations in axial and temporal resolution, the analysis of Myc dynamics implied an additional drawbacks relying on the lack of cell division reporters and the use of GFP-MYC nuclear signal, which is maintained intentionally at very low physiological levels and is distributed unhomogeneously throughout the nucleus. There are tracking packages that allow lineage reconstruction which rely on nuclear segmentation and thus require homogeneous nuclear signal and therefore lack information on cellular contacts (16, 27, 29), being inappropriate for the application in hand. Analysis based on cellular membranes is absolutely necessary for the identification of cell-cell contacts, which is a prerequisite for the reconstruction of neighbour relationships necessary for the study of cell competition. Thus, our analysis required automatic cell segmentation and

tracking based on both membrane and nuclei signals, being the nuclear signal dim, non-homogeneous and subject to intensity fluctuations. Several packages can perform lineage and neighboring analysis based on membrane signal (17–19, 21, 22, 25). However, some of these packages either do not provide an automatic tracking from those membrane images (18, 21, 25), or require highlighting of dividing cells (19). There are tracking methods that provide cellular based analysis such as EDGE-D (17) which does not handle cell lineage tracing across cell division, or MARS-ALT (22) which requires scripting and does not offer the integrated visualization of lineage trees, fluorescence marker and neighbourhood features. Therefore, our image analysis pipeline required several features over existing methods in order to allow the automatic tracking of cells with nuclear intensity fluctuations and strong limitations in axial and temporal resolution not relying on homogenous nuclear staining or mitosis highlighting, and for providing the integration of neighbourhood and lineage relationships with tree visualization tools.

We deployed strategies based on the definition of extended forms of spatio-temporal connectivity rules in order to overcome limitations in axial resolution and in Z and time connectivity (Fig. 1). Complex segmentation approaches based on 4D mathematical morphology operators (20, 33) were discarded for the analysis of mESC due to their sensitivity to spatio-temporal image resolution (20) leaving data association methods (16, 18) as the best option to handle this analysis. Moreover, the necessity of managing non-highlighted mitosis prevented the use of tools that do not directly handle actively dividing cells (17) or relying on highlighted mitotic spindles (19) and led to the requirement to manage cell segmentation based on feedback from the time dimension. This precluded the use of software based exclusively on

segmentation in a time-independent manner, such as MARS (22), ACME(21) or RACE (18), and prompted us to implement a cell segmentation algorithm that was updated receiving feedback from tracking, and took into account biological knowledge such as minimum temporal distance between two divisions or nuclear shape before mitosis. To establish a comparative study of the performance of ESC-T with respect to a tracking solution based on nuclear staining, we applied the ImageJ plugin TrackMate (26, 27). The gold standard was generated by manual segmentation and tracking of an image subset of 12 frames (1.4h time lapse) of a colony of 14 cells. Segmentation and tracking accuracy was evaluated for the results obtained with TrackMate (considering the spherical detection as segmentation), ESC-T and manually curated ESC-T (ESC-T + corrections). Segmentation (SEG) and tracking (TRA) metrics were computed to evaluate accuracy of the different methods using the software provided by the challenge (12) (detailed in Supplementary Materials and Methods), thus revealing superior performance of ESC-T as compared to TrackMate (Fig. 4a). Evaluation of execution times was carried out by measuring computation times with respect to number of frames as described in Supplementary Materials and Methods. Execution times varied from 3.6 minutes to 10.23 hours (Fig. 4b), being the complexity of the algorithm O(n); i.e. linear-time with respect to the number of video frames (Pearson's correlation $R>0.99$, $p<0.001$).

ESC-T was additionally used to study the changes occurring during mitosis, when cells undergo a drastic cell rounding which is thought to facilitate organization within the mitotic cell and be necessary for the geometric requirements of division (30–32). These cell shape changes were recapitulated when plotting the distribution of cell roundness as a function of distance to mitotic completion (Fig. 4c). ESC-T analysis showed the expected increase in cell roundness

(defined in Supplementary Table_S1) occurring during mitosis, values tending to 0 (highest roundness) as cells approached the cell division (0 distance to mitosis), in contrast to the stable distribution of roundness as the elapsed time before and after the mitotic event increased. Myc levels determine ESC proliferative ability (34), and we have previously reported that endogenous cell competition refines the epiblast cell population through the elimination of cells with low relative Myc levels (35). ESC-T has allowed us to establish the biological role of Myc-related endogenous cell competition, and find a correlation between GFP-MYC levels and ESC proliferative capacity (manuscript in preparation). To investigate whether the cell's proliferative capacity is influenced by the expression of Myc in neighbouring cells, the levels of GFP-MYC intensity of neighbours were plotted as a function of distance to cell birth in cells classified as dividing and non-dividing according to their live-recorded data (Fig. 4d). This analysis revealed no significant differences between dividing and non-dividing cells during the cell lifetime, thus demonstrating that proliferation potential of ESCs is not influenced by Myc intensity levels of neighbouring cells. ESC-T capabilities for studying cell motility in mESC cultures are highlighted in Figure 4e-f. Cell tracks of a random selection of cells were plotted along with their directionality indexes (Fig 4e, f). The directionality indexes were computed for the cells classified as dividing and non-dividing showing no significant differences between these two groups of cells, as expected (Fig. 4g). Directionality index was studied in the whole cell population tracked in the context of the cell lifetime, revealing a decrease in directionality as a function of lifetime (Fig. 4h). A higher directional movement is observed at short lifetimes, which decreased as lifetimes prolonged, eventually reaching a horizontal asymptote. This negative correlation is most likely related to the number of frames analysed rather than the

lifespan of the cell and highlight the strong influence that the observation time exerts in directionality analysis. The plot allows an estimation of the tracking time at which the curve stabilizes to the horizontal asymptote which is at lifetimes around (17 hours) 150 frames. This represents the minimum observation time required to identify motion as directionally biased and to capture a reliable quantitative estimation of its persistence.

ESC-T allowed us to analyse Myc intensity fluctuations in relation with lineage and neighbourhood history in mESC cultures expressing GFP-MYC and tdTomato imaged by confocal microscopy during a 24 hour period of time. ESC-T proved to be a very powerful tool for studying morphological changes occurring during mitosis, exploring if the proliferative ability of cells is affected by the Myc levels of neighbouring cells, and analyzing mESC motility related parameters in correlation to Myc expression. Moreover ESC-T has led us to uncover Myc-driven endogenous cell competition mechanism in stem cell pluripotency maintenance (manuscript in preparation).

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ESC_Track software package and complete instructions for its use are freely available at www.cnic.es/en/investigacion/2/1186/tecnologia.

Competing Interests

The authors declare no competing interests.

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Fig. 1. Segmentation and tracking computational pipeline.

Fig. 2. Lineage tree representation generated by ESC-T and visualization of cell trajectories, contacts and their relationship to Myc expression. a) Assignment of cell identifiers (o_ID) to every tracked cell to allow dividing cells to be connected with their progeny and represented as a lineage tree; the example shows the lineage tree of progenitor 2 at 5 different time-points at which every cell entity is represented as rectangle in which cellular IDs are detailed. **b-d**) Lineage tree representation of extracted cellular features during tracking times (min). For every cell of the tree at each time-point, a selected feature is represented as a color coded rectangle to visually highlight changes in the feature along chronological lineage traces. Panels show nuclear GFP-MYC intensities **b),** nuclear maximum GFP-MYC intensity of neighbors **c),** and nuclear volumes **d)** for each cell in the lineage tree using the color code shown to the right of each chart. **e)** Representation of motion tracks and Myc expression in 2 selected cells; the figure depicts trajectories and color-coded MYC-GFP normalized intensity values obtained at each time-point using ESC-T. Cell coordinates at each time point, with squares color-filled according to the corrected mean_compensated_Mean_Myc score for the nucleus. Mitotic events and starting and final locations are represented by triangles, circles, and diamonds, respectively. Trajectories are highlighted in green and blue. **f)** Video stills (supplemental Movie S1) obtained at the indicated time points, combining the Myc expression levels and trajectories as in **(e).**

Fig. 3. Z-stack confocal images from mESC 4D image datasets, showing limitation in x, y, and z resolution. mESCs expressing GFP-MYC and tdTomato were imaged by confocal microscopy with Z sections spaced at 2 µm. Images show X-Y views of green (GFP-MYC) and red (tdTomato) signals, with the selected nuclei highlighted yellow (**a)** and blue (**b)**. **c)** XZ projection of 3D Image Z-stack and segmentation overlap for the nuclei highlighted in **a** and **b** (yellow and blue, respectively).

Fig 4. ESC-T performance evaluation, cell shape, neighbour GFP-MYC level and motion analysis after *in silico* synchronization to cell division time using ESC-T. **a)** Evaluation of TrackMate and ESC-T with and without manual corrections in terms of execution time (min.), segmentation (SEG) and tracking (TRA) accuracy. Estimated times from an expert user have been included for the complete manual segmentation and tracking (manual time reference), and the correction in ESC-T + correction. **b)** Computation times (in hours) for the segmentation and tracking of videos

406 from 12 to 412 frames with 14 cells in average. **c)** Distribution of cell *roundness* (Table S1) as a function of time to
407 mitosis (min.), obtained from live-recorded data; showing an increase of *roundness* during mitosis ($p<0.001$). **d)**
408 Distribution of GFP-MYC levels of neighbors (MeanMyc_Neighbours_Compensated, Table S1) after *in silico*
409 classification of ESCs into dividing (red) and non-dividing (blue) according to live-recorded data as a function of
410 distance to the beginning of each cell track; showing no significant differences between dividing and non-dividing
411 cells ($p>0.001$). Locally weighted scatterplot smoothing has been applied in **(c)** and **(d)**. **e)** Cell tracks of 6 randomly
412 chosen cells aligned at the origin of live recording time. **f)** Analysis of the motility related parameters mean velocity
413 and directionality index (effective_distance/walking_distance), corresponding to the individual cell tracks presented
414 in **(e)** (color coding matches individual cell tracks). **g**) Directionality index after in silico classification of cells as
415 dividing or non-dividing; showing no significant differences between dividing and non-dividing cells ($p>0.001$) **h**)
416 Directionality index with respect to the (image-based) lifetime of every cell analyzed in a live cell recording
417 experiment. Locally weighted scatterplot smoothing has been applied.

418