Live imaging genetically-encoded fluorescent proteins in embryonic stem cells using confocal microscopy

Jérôme Artus and Anna-Katerina Hadjantonakis*

Developmental Biology Program, Sloan-Kettering Institute, New York, New York 10021, USA

The ability to non-invasively visualize, track and quantify events as they take place in living cells is essential for developing a deeper understanding of biological processes. The recent explosion in the field of stem cell biology, and the therapeutic potential of embryonic and adult stem cells have necessitated the development of a suite of tools for live imaging stem cells. ES cells are derived from the inner cell mass of blastocyst stage mammalian embryos. They share many characteristics between different organisms in that: (1) pluripotent ES cells can be maintained *in vitro* in an undifferentiated (stem cell) state indefinitely without undergoing senescence, and (2) they can be differentiated into multiple cell types both *in vitro* and *in vivo*. ES cells represent a powerful model to study: (1) the molecular network coordinating stem cell self-renewal vs. differentiation, (2) the early development of mammalian embryos, and (3) the directed differentiation into specific cell types for cell-based therapies. In this review we introduce some of these topics, and highlight current live imaging techniques applied in ES cells.

Keywords embryonic stem cell; mouse; live imaging; fluorescent protein; GFP; RFP; laser scanning microscopy; confocal; time-lapse; dynamics

Abbreviations BMP – Bone Morphogenic Protein, ChIP – chromatin immunoprecipitation, DsRed – *Discostoma sp* red fluorescent protein, ES – embryonic stem, ECFP – enhanced cyan fluorescent protein, EGFP – enhanced green fluorescent protein, EYFP – enhanced yellow fluorescent protein, FACS – fluorescence activated cell sorting, FCS – fluorescence correlation spectroscopy, FLIM – fluorescence lifetime imaging, FRET – Forster or fluorescence resonance energy transfer, GFP – green fluorescent protein, ICM – inner cell mass, LSM – laser scanning microscopy, PE – parietal endoderm, RFP – red fluorescent protein, QD – quantum dot, TE – trophectoderm.

ES cell self-renewal and pluripotency

Embryonic stem (ES) cells were first isolated from mouse embryos [1, 2]. They are derived from the inner cell mass (ICM) of preimplantation embryos at the blastocyst stage, but have been also isolated from earlier eight-cell stage embryos [3] and morulae [4]. ES cells possess several unique properties. They can be propagated indefinitely *in vitro* in an undifferentiated (stem cell) state and retain a stable karyotype. They can be differentiated both *in vitro* and *in vivo* into cells representing the three germ layer (ectoderm, mesoderm and endoderm) derivatives (**Fig. 1**). Interestingly, when injected into a recipient blastocyst stage embryo, ES cells contribute efficiently to the developing embryo and resulting animal. Notably ES cells will contribute to germ cells, which will go on to form the germ line. Thus, the pluripotent state of ES cells offers unprecedented opportunities, and is harnessed when genetic modifications are engineered into ES cells to create genetically modified mice [5].

To date, ES cell or ES cell-like cells (as no germline transmission has been reported) have been isolated from numerous mammals including for example bovine [6-11], equine [12], hamster [13], human [14], mink [15], monkey [16], pig [17], rat [18], and sheep [17]. Interestingly, ES cells have been also isolated from non-mammalian species including chickens, quails [19] and zebrafish [20-23] where germline transmission has been reported. The development of efficient methods to derive and

* Corresponding author: e-mail: hadj@mskcc.org

genetically-modify ES cells from various organisms is expected to have a profound impact not only in biology, but also in medicine and agriculture.

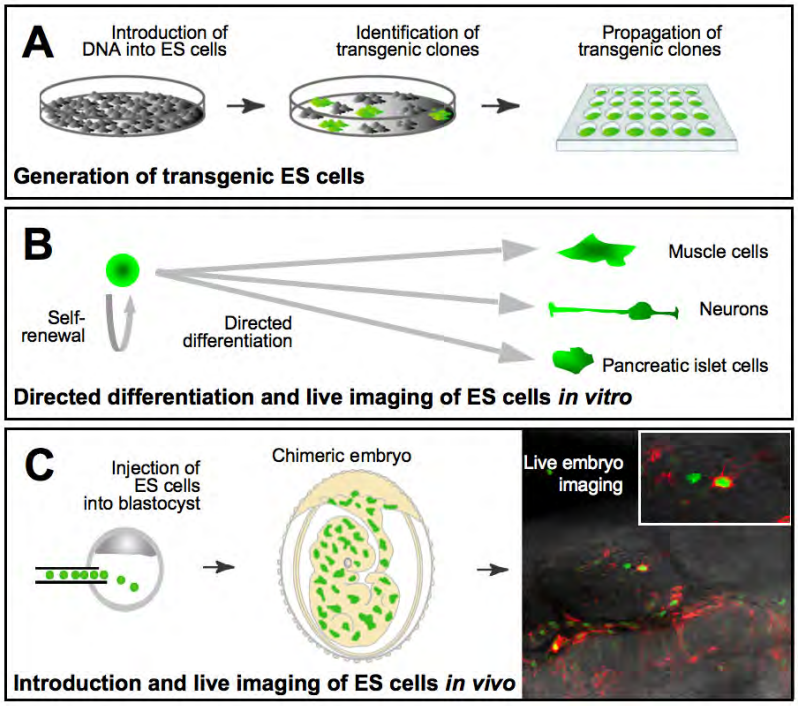

Figure 1. Transgenic ES cells and their differentiation *in vitro* and *in vivo*. A: Schematic representation of the sequential steps required for the generation of transgenic ES cell clones. B: ES cells have the dual capacity to self-renew and differentiate into specific cell types *in vitro* depending on culture conditions. C: ES cells can be re-introduced *in vivo* into the context of an embryo or adult animal. To do this, ES cells are microinjected into a blastocyst stage embryo resulting in the generation of a chimeric embryo. Such an embryo is reintroduced into the uterus of a surrogate female and allowed to develop to the required embryonic stage, or to term. Embryos can be isolated, cultured *ex utero* and live imaged to obtain information on ES cell derivative cell types at sub-cellular resolution *in situ*. This type of data is depicted on the far right of panel C. In this experiment transgenic ES cells co-expressing myristoylate-RFP (labeling the plasma membrane) and histone H2B-GFP (labeling active chromatin) fusions were introduced into a non-transgenic embryo. The embryo was isolated (i.e. dissected free of the maternal uterus) at midgestation and cultured *ex utero* on the stage of a confocal microscope.

Extrinsic and intrinsic factors mediating self-renewal and pluripotency

A large body of work has followed the initial isolation of murine ES cells over a quarter of a decade ago ([1, 2]; for a historic review see [24]). Most of our knowledge of ES cell biology comes from the study of the molecular mechanisms supporting pluripotency in mouse ES cells, and more recently work performed investigating human ES cells. Mouse ES cells are usually cultured on fibroblast feeder cells [25]. One key factor required for stem cell maintenance is Leukaemia Inhibitory Factor (LIF), a member of IL-6 cytokine family [26, 27]. Despite its essential action through the LifR/Gp130/Stat3 pathway (for review see [28]), addition of recombinant LIF protein to cell culture media is not sufficient to isolate and propagate ES cells in an undifferentiated state suggesting that some others external factors are required, which are likely to be present in the serum [29], which is a necessary constituent of the culture media. Recently, Ying and colleagues demonstrated that mouse ES cells can be maintained in defined media that only contains exogenous LIF and BMP (Bone Morphogenic Protein) [30]. Several reports demonstrate that Wnt signaling pathway also plays an important role in maintaining pluripotency. For example, Wnt activation by pharmacological inhibition of GSK3 is able to maintain both murine and human ES cells

[31]. More recently, it has been shown that Wnt pathway acts synergistically with LIF in murine ES cells [32].

Dissecting molecular mechanisms in murine ES cells can be useful to better understand the properties of human ES cells. However, it should be noted that some important differences exist between ES cells of different species. For example, the presence of LIF is not necessary for ES cell propagation from equine [12], human [14] or rhesus monkey [16]. Moreover, whereas exogenous BMP promotes self-renewal of mouse ES cells, human ES cells differentiate into trophoblast [33]. Thus, it appears that many signaling pathways have a dual action in promoting patterning and differentiation during embryonic development and maintaining pluripotency. This apparent discrepancy probably reveals that pluripotency is in fact an unstable equilibrium, and in doing so provides a cell with two main advantages: (1) a global and mutual inhibition of differentiation, and (2) a high sensitivity to minor alterations in their environment which can trigger differentiation.

A core-regulatory network of transcription factors

Pluripotency is sustained by a core regulatory network of transcription factors, that act both to promote self-renewal and inhibit differentiation. Three key molecular players associated with pluripotency have been identified to date, they include: (1) the POU domain-containing factor OCT4, (2) the HMG domain-containing factor SOX2, and (3) the homeobox domain-containing factor NANOG. Each of these genes has been shown to be essential for the maintenance and propagation of ES cells and for early embryonic development in the mouse. Indeed, *Oct4* inactivation in mice leads to a lethality just after implantation, and is characterized by a loss of pluripotency of the ICM which differentiates into trophectoderm (TE) [34]. *Sox2* deficient mouse embryos exhibit profound defects in the ICM-derived epiblast [35]. Isolated ICMs lost their pluripotency and differentiated into TE. Lastly, mouse embryos lacking *Nanog* have no discernable epiblast at periimplantation stages and isolated ICM differentiated in culture into parietal endoderm (PE) [36]. Thus, both *in vivo* experiments using genetically modified (knockout) mice, and *in vitro* experiments where these factors are knocked-down or overexpressed, support the idea that these three factors represent the core component of a network required for the maintenance of pluripotency.

Indeed, OCT4 and SOX2 can directly interact with each other [37] and in combination with NANOG generate positive feedback loops which regulate their own expression [38]. Extensive analysis by chromatin-immunoprecipitation (ChIP)-on-chip or -PET respectively in human [38] and mouse ES cells [39] reveals that these factors can co-occupy multiple sites, acting both to promote the expression of genes important for self-renewal, but also to block the expression of genes implicated in differentiation. These results, in combination with gene expression profiling using microarrays on murine ES cells in which *Oct4* expression was tightly controlled, provide strong evidence for the primary target genes regulated by OCT4 [40]. Furthermore, using fluorescence resonance energy transfer (FRET) analysis in live ES cells, through the generation of fusion proteins to enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), Niwa and colleagues demonstrated that OCT4 can also interact with CDX2 (a gene expressed widely in early embryos but later restricted to the TE), and forms a repressive complex of their respective target genes [41]. In conclusion, it appears that a precise combination of these transcriptional regulators is required to sustain pluripotency through a complex network not only through gene expression but also by regulation of nuclear protein dosage through nuclear import selective mechanism [42] and regulation of protein activity through post-translation modification such as sumoylation [43, 44].

The development of fluorescent reporters in ES cells (**Fig. 1A**), by transgenesis or targeted mutagenesis of genetically-encoded fluorescent proteins, whose expression parallels endogenous gene expression or which are constitutively active highlighting dynamic subcellular compartments, will be essential for future work. For example, such reporters can be used to study the dynamic changes in both cell morphology, division and death in addition to gene expression under differentiating or non-differentiating (i.e. stem cell) conditions, but also such reporters can be incorporated in large-scale screens to identify new factors required for these processes. To date, several studies used a *Nanog*-EGFP

[45] and an *Oct4*-EGFP [46] transgenes, and an EGFP knock-in into mouse *Sox2* locus [47] have been reported.

Transmitting pluripotency to differentiated nuclei

Cell fusion experiments have revealed that the pluripotent state associated to both murine and human ES cells can be transmitted to differentiated somatic cell nuclei [48-50]. The nature and mechanism of action of the nuclear reprogramming factor(s) is still unclear. However, recent data suggest that a combination of several factors can reprogram differentiated cells into a state equivalent to ES cells (i.e. exhibiting pluripotency). Indeed, Yamanaka and colleagues showed that co-expression of four transcriptional regulators: *C-myc*, *Klf4*, *Oct-4* and *Sox2,* in fibroblast cells can dedifferentiate them into a pluripotent state that is compatible with a significant and efficient contribution to embryonic tissues including the germ line of chimeric animals [51-54]. Gaining a better understanding of how reprogramming is achieved should expedite the development of new ethically acceptable strategies for the use of ES cells and/or their characteristic features in regenerative medicine.

ES cells as a model for cellular differentiation

The pluripotency of ES cells, their ease of manipulation in culture, and their ability to contribute to the mouse germline, has not only facilitated a plethora of strategies for genetic modifications at base pair resolution (See for examples [55-57]), but has also provided a model of differentiation both *in vitro* and *in vivo* (**Fig. 1B-C**). Since ES cells are introduced into preimplantation stage embryos they can efficiently contribute to all three embryonic germ layers, but poorly to extraembryonic tissues [58], it is possible to use this experimental approach to follow different developmental processes as they take place *in situ* in wild type *vs.* mutant or perturbed contexts. Furthermore if ES cells are genetically marked their progeny can be live imaged *in situ* (**Fig. 1C**).

In vitro, when ES cells are cultured in the absence of feeders and LIF, and in presence of diverse chemical compounds, their differentiation can be directed to give rise to various cell types: from neurons [59, 60], to germ cells [61]. Thus, they can be useful for regenerative tissue purposes. In this context, it appears important to define optimal medium conditions to drive ES cell differentiation into a particular cell type (see for review [62]). Indeed the field of live ES cell imaging is of great interest to the pharmaceutical and biotechnology industries, and many are now developing high-throughput screening platforms for automated analysis of intracellular localization and dynamics. ES cells have also provided a powerful genetic model to study the implication of key genes implied for the formation of the first cell lineages (epiblast, primitive endoderm and TE), dissecting in greater detail the actual picture of early developmental events (see for review [63]).

Live imaging: methods and probes applied in ES cells.

As a principle methodology in the field of cell biology, optical microscopy has been exploited for the study of ES cells since their initial derivation. Indeed, fluorescence imaging remains an essential component of ongoing studies aimed at understanding the molecular hierarchies used to control ES identity and behavior. Since it is widely recognized that observing processes as they take place *in situ* (within living cells or embryos) adds a vital extra dimension to our understanding of cell fate and behavior, efforts are underway to incorporate live imaging methods to the study of ES cells. The advent of fluorescent labeling methods combined with the plethora of sophisticated light microscope techniques make studying dynamic processes in living cells almost commonplace, and live imaging is being increasingly applied in ES cells. Therefore continued efforts in the development of improved imaging tools will likely move the stem cell field forward, and facilitate the development of a deeper and dynamic understanding of self-renewal and differentiation.

The advent of Green Fluorescent protein (GFP) from the jellyfish *Aequoria Victoria* [64, 65] and its spectral variants as stable and bright vital fluorescent probes, in addition to other genetically-encoded

fluorescent proteins, most notably the red fluorescent protein (RFP) DsRed from the sea anemone *Discostoma sp* [66-68], have catalyzed a revolution in non-invasive live cell imaging. These fluorophores allow complex biochemical processes to be correlated with functioning of proteins in living cells. Native enhanced green fluorescent protein (EGFP), and proteins tagged with EGFP (or other genetically-encoded fluorescent proteins) can be visualized in cells over long periods of time and with minimal photobleaching (the photo-induced destruction of a fluorophore). Several GFP and DsRed variants have been shown to be developmentally neutral and amenable to use in ES cells and mice thereby paving the way for live imaging experiments in ES cells [69-71]. Such fluorescent proteins can be introduced into ES cells through standard transgenesis or targeted mutagenesis methods (**Fig. 1A**). Beyond the scope of this review the emergence of new small molecule fluorescent dyes, nanocrystals (quantum dots) and genetically-encoded tags that can be coupled with fluorochromes (e.g. molecular beacons) complement the virtual spectrum of available genetically-encoded fluorescent proteins (**Fig. 2C**) that can be applied in ES cells, and are pushing the envelope for types of live imaging experiments that can now be performed [72].

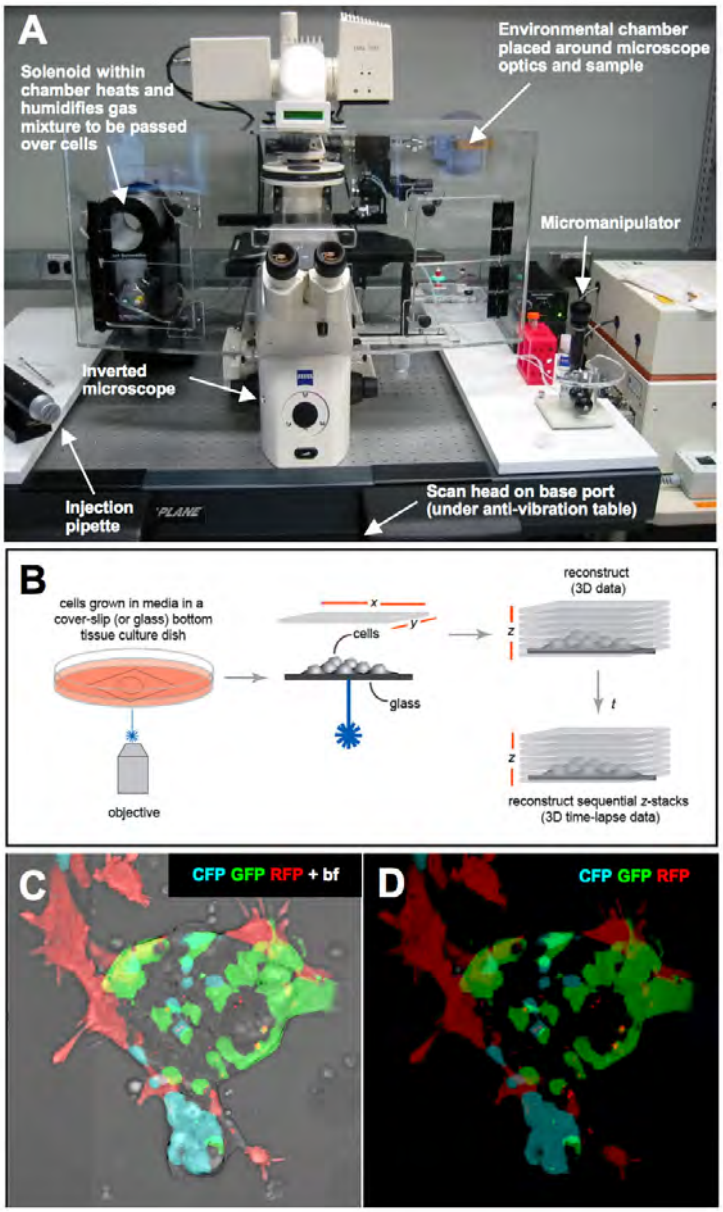

Figure 2. Live imaging of ES cells. A: Laser scanning confocal microscope set-up dedicated to live imaging of ES cells and mouse embryos. B: Schematic representation of the arrangement of a glass bottomed tissue dish and microscope objective. *z*-stacks of confocal *xy* images are acquired at timed intervals to generate 4D (3D time-lapse) data. C and D: 3D reconstructions of laser scanning confocal data of a mixed ES cell colony containing a wild type cell line and three different transgenic cell lines expressing either CFP, GFP or RFP. Images are presented overlayed on brightfield image (bf) in panel C, or dark field alone, in panel D.

When placed under the control of a gene and/or cell type specific promoter, genetically-encoded fluorescent proteins act as vital cellular markers that reflect states of gene activation and/or silencing or mark a specific population of cells (e.g. those that retain pluripotency through expression of *Oct4*), and can be used to draw correlates with gene expression, cell behaviors (e.g. cell movement) and/or lineage specification (e.g. TE *vs*. ICM of the mammalian blastocyst). Furthermore fluorescent proteins such as GFP and its variants can be fused to virtually any protein of interest to analyze subcellular localization of a protein of interest and its dynamic redistribution within a living cell. Indeed fluorescent proteins have been used as tools in numerous applications, for example as minimally invasive markers to track and quantify individual or multiple protein species, as probes to monitor protein-protein interactions (for example using FRET), as photo-modulatable (photoactivatable or photoconvertible) proteins to highlight

the fate of specific protein populations within a cell, or specific cells within a cohort for fate mapping studies, and as biological sensors to describe signaling pathway activation [73-75].

When fused to other proteins fluorescent proteins can be used to label subcellular compartments such as the nucleus (through fusion to histones or nuclear localization sequences), the secretory pathway including the plasma membrane (through use of lipid modified fusions) and cytoskeletal elements (including microtubules through fusion to tubulin or the tubulin associated protein tau, actin filaments through fusion to actin or actin binding proteins, and intermediate filaments through fusion to vimentin). Most notably, fusions to human histone H2B have proven useful for studying cell division, as this fluorescent fusion marks sub-nuclear, in fact chromosomal, dynamics during mitosis, which when combined with spectrally-distinct plasma membrane fusions facilitates the simultaneous imaging of cell morphology (**Fig. 3**) and provides a dynamic equivalent of routine histology [76-78]. Furthermore, nuclear localized fusions such as the H2B-GFP allow image segmentation, identification and tracking of individual cells within a cohort.

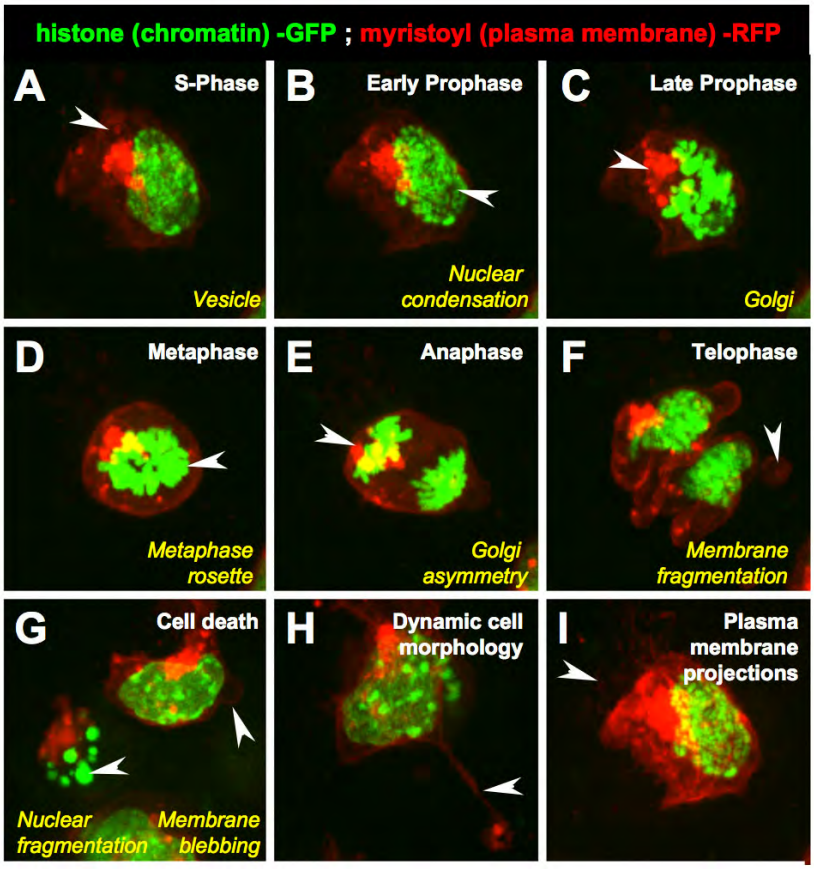

Figure 3. Dynamic 4D (3D time-lapse) imaging of transgenic ES cells expressing spectrally-distinct subcellularly localized genetically-encoded fluorescent proteins. A-F: time-lapse sequence of ES cells expressing two fluorescent protein transgenes. The morphology of the cells (revealed with RFP) and the arrangement of chromatin (revealed with GFP) can be followed as mitosis progresses through its stereotypical sequential phases. Dynamic changes in both the morphology (rounding up) and chromatin distribution (as chromosomes condense) can be visualized in living cells as they enter M-phase of the cell cycle and undergo cell division. Characteristic features of cell death such as nuclear fragmentation and plasma membrane blebbing can also be imaged in live cultures (panel G), as can dynamic changes in cell morphology and plasma membrane projections (panels H and I).

New and improved fluorescence imaging methods have been essential to studies examining the localization and kinetic behavior of GFP-tagged proteins in living cells [74]. These techniques include 4D microscopy (3D time-lapse imaging – **Fig. 2B**), fluorescence resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), fluorescence lifetime imaging (FLIM), and fluorescence correlation spectroscopy (FCS). Notable among these with respect to its application in ES cells and embryos is 4D microscopy. Here, time-lapse observations of fluorescent molecules are collected as three dimensional (3D) data (*z*-stacks of *xy* images – **Fig. 2B**) rather than one image in a single focal plane. This allows information to be gained on the behavior of individual cells in time and space. It represents a step forward from the routine, but static, histological methods that have previously dominated the field.

An additional consideration is speed of data acquisition, particularly when multiple fluorophores are imaged simultaneously, a single probe is analyzed ratiometrically, or when events being investigated are transient and highly dynamic (e.g. Ca^{2+}). In such cases high-speed and high-resolution confocal microscopes with multispot (e.g. using a spinning disc confocal) or slit-scanning excitation and/or detection capabilities provide improved temporal resolution over conventional laser scanning confocal methods (**Fig. 4**). This type of high-speed approach has revolutionized our ability to visualize intercellular and subcellular molecular interactions in real-time. Often, computational analysis of 4D image data allows the extraction of quantitive parameters including velocity distributions, volumes of structures and signal intensities that can for example be correlated to directional cell movement, cell division and death, and gene expression within a cell.

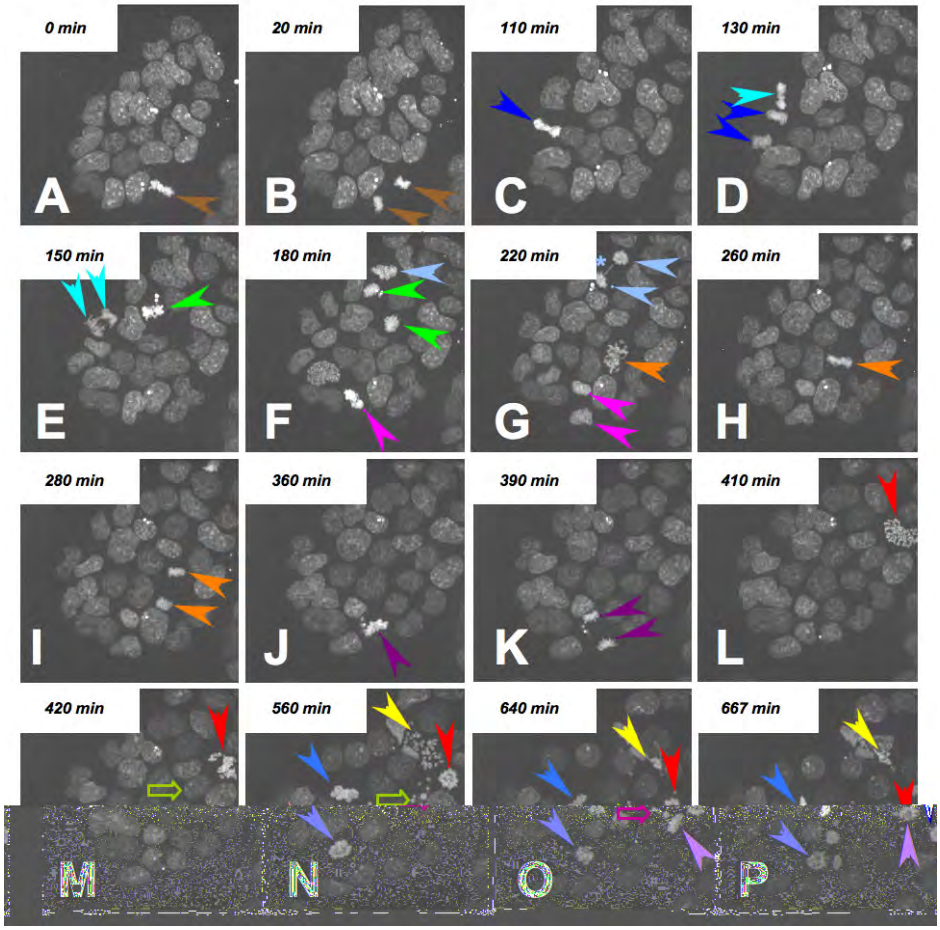

Figure 4. ES cells are sensitive to photodamage. The density of data acquisition (*z*-stack size and density, and time-lapse interval), laser intensity and other variables (e.g. binning if offered by the software package used) need to be optimized for each type of experiment. This set of panels is taken from a 4D (3D time-lapse) experiment of ES cells exhibiting constitutive expression of an H2B-GFP fusion transgene which labels active chromatin including condensing chromosomes. Each panel represents a 3D reconstruction of a *z*-stack. There are twenty-six ES cells in the main cluster presented in the field of view, with one being in mitosis (at metaphase) at the start of the time-lapse sequence (panel A = 0 min). At least seven additional cells divide over the following period of 390 minutes (= 6.5 hours). Dynamics of abnormal mitotic progression can be seen after approximately 4 hours. For example a lagging chromosome (asterisk) is seen at anaphase in one dividing cell (at 220 min). By 410 minutes one cell (red arrowhead) is seen to enter prophase, but the arrangement of its chromosomes is aberrant as it progresses through mitosis into metaphase (at 420 minutes). Over the next 247 minutes the chromosomes of four additional cells are seen to condense as these cells appear to initiate mitosis, but they fail to progress beyond prophase or metaphase. Furthermore in the second half of the experiment (360 min onwards) a change can be seen in the shape of individual nuclei which appear to adopt a more rounded morphology, additionally nuclear fragmentation can be seen in some cells (green open arrow). Colored arrowheads highlight individual cells undergoing mitosis in consecutive panels. The 3D time-lapse sequence was acquired on a spinning-disc confocal using a 63x PlanApo NA1.3 objective, and is composed of *z*-stack projections of 72 *xy* sections acquired at 0.35µm intervals every 10 minutes.

Regardless of imaging technique used it is crucial to consider the cells' health on the microscope stage during the course of the experiment. ES cells are sensitive to photodamage (**Fig. 4**), particularly in the presence of fluorophores (which generate free radicals upon photobleaching). Furthermore on-stage culture conditions should mimick those within an incubator, and it is important to keep the cellular

environment constant. To this end dedicated microscope set-ups are often used for live imaging of ES cells and embryos (**Fig. 2B**).

Conclusions

ES cells are an immensely powerful biological tool in several fields including developmental biology, regenerative medicine and oncogenesis. In this review, we have focused on how the rainbow palette of genetically-encoded fluorescent proteins combined with the development of new methodologies in visualization, acquisition and data analysis are being used to decipher the properties of ES cells. Microscopic observation has always been a pivotal methodology in cell and developmental biology, both for determining the normal course of events and for contrasting with the results of experimental and pathological perturbations. Recent advances in optical imaging techniques and genetically-encoded fluorescent protein probes are converging to provide not only enhanced resolution, but also dynamic assays for studying cellular and subcellular events in living cells. These advances will help bridge the gap between static observations made in fixed specimens and dynamic observations using live cell imaging.

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