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Review

Gene expression within a dynamic nuclear landscape

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Molecular imaging in living cells or organisms now allows us to observe macromolecular assemblies with a time resolution sufficient to address cause-and-effect relationships on specific molecules. These emerging technologies have gained much interest from the scientific community since they have been able to reveal novel concepts in cell biology, thereby changing our vision of the cell. One main paradigm is that cells stochastically vary, thus implying that population analysis may be misleading. In fact, cells should be analyzed within time-resolved single-cell experiments rather than being compared to other cells within a population. Technological imaging developments as well as the stochastic events present in gene expression have been reviewed. Here, we discuss how the structural organization of the nucleus is revealed using noninvasive single-cell approaches, which ultimately lead to the resolution required for the analysis of highly controlled molecular processes taking place within live cells. We also describe the efforts being made towards physiological approaches within the context of living organisms.

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Introduction

Gene expression encompasses the launching of a series of molecular pathways enfolded within structural changes occurring in nuclear architecture, and resulting in the transcriptional onset at specific gene loci. For years, these pathways have been exhaustively examined using biochemical and molecular approaches without much consideration of the special restrictions presented by the nuclear architecture. Current methodologies for tracking molecules spatially and temporally by means of fluorescent tagging have been put to use in the analysis of the gene expression pathway as it occurs *in vivo*. A coherent view of gene expression requires the knowledge of the molecular players involved, together with the understanding of the biophysical and structural cellular milieu in which they perform. Here, we give an overview of our current understanding of gene activation taking place within the context of nuclear structure and originating particularly from time-lapse analysis performed in living cells.

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The flow of gene expression

Let us portray the process of gene expression by roughly sketching the main occurrences taking place at a specific gene locus destined to undergo gene activation. We tend to describe genes in either silenced or active states. Although it was perceived that silenced genes are to be found in condensed heterochromatin, while expressed genes are located in open euchromatin areas, it seems that this is not the complete picture. In fact, genome organization is more complex than such a bimodal depiction of chromatin packaging states (Gilbert *et al*, 2004), and we still do not fully understand all the factors that govern gene activation and silencing (Spector, 2004). The repressive state is thought to be maintained by a series of particular but reversible biochemical modifications occurring on the histone proteins, which form the nucleosomes (Jenuwein and Allis, 2001). The onset of the transcriptional process requires the biochemical dismantling of the silenced structures, which occurs via a counteracting series of modifications taking place on the histone proteins. This transition is still not conceptually well understood, but it enables DNA to become accessible to transcription factors and sets the groundwork for the assembly of the transcriptional machinery on the gene of interest. The RNA polymerase II enzyme can proceed from an initiating state into an elongating state and processively translocate along the DNA to synthesize an RNA transcript. The RNA molecule forms an RNP (RNA–protein complex) while transcription is proceeding (Dreyfuss *et al*, 2002; Maniatis and Reed, 2002; Neugebauer, 2002). At the end of one round of the transcriptional process, the RNP and the polymerase detach from each other and from the DNA. This process can commence to produce high or low copies of RNA depending on the regulation of this gene. The RNP must then travel through the nucleoplasmic space, encountering enroute numerous nuclear structures, to reach the port of nuclear exit at the nuclear membrane and to translocate into the cytoplasm where RNA is translated into protein. Clearly, just this description of occurrences, as they proceed from a nuclear located gene

towards the cytoplasm, leads to a dynamic view of the flow of gene expression. The extensive progress made in visualizing these processes in living cells has brought upon an additional level of complexity in the comprehension of gene expression dynamics (Misteli, 2001; Darzacq *et al*, 2005). We now realize that nuclear constituents are constantly mobile, and that each molecule, even large structures as chromatin DNA, have unique kinetics. Therefore, correct timing in combination with correct positioning in space is a necessity in coordinating the processive act of expression. We now proceed to show how this understanding of nuclear dynamics has taken form.

Chromosome dynamics

While it is clear that chromosomes occupy defined nuclear territories and do not significantly overlap (Manuelidis, 1985; Cremer and Cremer, 2001), whether some regions in the nucleoplasm are more adapted for gene expression than others is still a matter of debate (Pederson, 2004). Transcriptional activation induces large-scale chromatin decondensation that can be observed directly on tandem gene arrays (Tsukamoto *et al*, 2000; Muller *et al*, 2001; Janicki *et al*, 2004). Specific loci in interphase cells are dynamic exhibiting different mobilities and positioning according to their integration sites (Heun *et al*, 2001; Chubb *et al*, 2002; Yamamoto *et al*, 2004) or their transcriptional activity (Volpi *et al*, 2000; Mahy *et al*, 2002; Williams *et al*, 2002; Chambeyron *et al*, 2005) (e.g. for DNA mobility see Supplementary Movie 1). Live cell imaging of chromosome motion (Gerlich and Ellenberg, 2003) allows very precise dissection of the succession of events and positions adopted by chromosomes in different situations. These methodologies could demonstrate that chromosome position may have an inherited component in cultured cells (Gerlich *et al*, 2003). On the other hand, a parallel study using different constraints concluded that interphase positions of chromosomes were not well maintained in daughter cells (Walter *et al*, 2003). It seems therefore that there is only a certain degree of maintenance of chromosome position during mitosis, and further studies will reveal whether there is organized control of such processes. In addition to mitosis, global chromatin dynamics during interphase have been studied using GFP-fused DNA binding proteins such as core histones or other nucleosomal components. Photobleaching studies have demonstrated that many of these proteins (e.g. H2B, H3, H4) are practically immobile and tightly associated with the chromatin fibers (e.g. see Figure 1; Lever *et al*, 2000; Misteli *et al*, 2000; Phair and Misteli, 2000; Kimura and Cook, 2001; Phair *et al*, 2004; Meshorer *et al*, 2006) (e.g. for chromosome dynamics see Supplementary Movie 2). Chromatin can undertake dramatic rearrangements during cell death and under specific stress. Fluorescent H2B has been used to demonstrate real-time fragmentation of the nucleus during apoptosis in culture, for following mitosis in living mice using intra-vital imaging (Yamamoto *et al*, 2004) and for showing the effects of energy depletion on intra-nuclear structures (Gasser, 2002; Shav-Tal *et al*, 2004a).

While particular findings report that the relocation of specific genes outside chromosome territories depends on their transcriptional activation, and while it seems clear that chromosomes adopt specific positions according to their gene density, the observation that RNA polymerase II enzyme is present in the whole nucleoplasmic space offering a homogenous distribution of local concentration points often termed 'transcription factories' (Jackson *et al*, 1998) tends toward a view in which transcription is not spatially restricted. RNA polymerase II is one of the most powerful molecular motors found in biological systems, and the simple action of transcription and movement of polymerases could easily drive DNA loci outside of their original position in the chromosome. Also, polymerases and other giant macromolecular complexes of the cell have a predicted tendency to associate in nonspecific entropy driven macrostructures (Marenduzzo *et al*, 2006), possibly explaining the relative

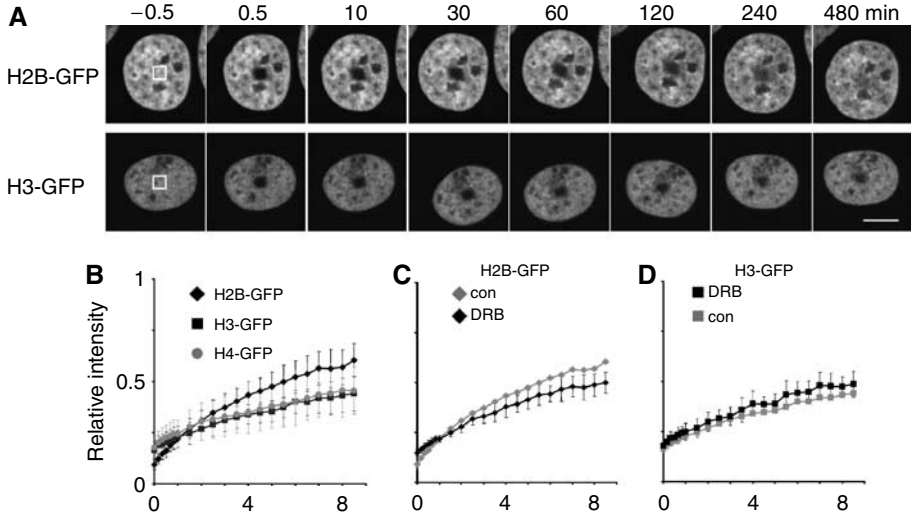

Figure 1 Measuring histone mobility by FRAP analysis. Different kinetic populations of histone-GFPs are revealed by FRAP. (**A**) A small area within the nucleus of a cell expressing histone-GFP was bleached, and confocal images were collected every 10 min for 1 h and every 30 min thereafter, in order to follow recovery of the signal within the bleached region. (**B–D**) Relative intensities (±s.d.; $n = 9$–22) within bleached areas were measured using images like those in (A). In some cases, the transcriptional inhibitor DRB (100 nM) was added 30–60 min before bleaching. Adapted and reprinted by permission from the Rockefeller University Press: *Journal of Cell Biology* (Kimura and Cook, 2001), copyright 2001.

immobility of transcription factories relative to translocating DNA molecules. Interestingly, it was shown that a number of genes that were contained on the same chromosome, but were separated by large chromosomal regions, had a strong tendency of sharing the same nuclear transcription space, thereby demonstrating controlled association of specific chromosome domains with sites of active transcription (Osborne *et al*, 2004). Indeed, a number of studies focusing on the positioning effects of certain gene loci during cell differentiation, a time at which cells undergo dramatic alterations in their gene activity patterns, have demonstrated the preferential positioning of genes in regard to each other or to the inner and outer regions of the nucleus (Kosak *et al*, 2002; Delaire *et al*, 2004; Kim *et al*, 2004; Brown *et al*, 2006).

RNA dynamics

Gene expression occurs simultaneously at multiple transcription factories in actively transcribing cells (Pombo *et al*, 2000). Therefore, at any given time, one can envision waves of mRNA transcripts moving from sites of transcriptions towards nuclear pores and destined to cytoplasmic translation. Some mRNAs diffuse in the cytoplasm until they encounter ribosomes (Fusco *et al*, 2003), while others are actively translocated on cytoskeletal filaments to ultimately localize at specific regions of the cell (Shav-Tal and Singer, 2005). The spatial sorting of RNA cargo in the cytoplasm requires the recruitment of specific motor proteins and the investment of cellular energy. However, what is the situation in the nucleus where transcripts originate? While no mechanism of active nuclear transport system is known to date, it has been provocatively suggested that a nucleoskeletal transport mechanism including nuclear motor proteins might exist. Indeed, the basic building blocks of the cytoskeleton, that is actin, nuclear myosin and a number of related proteins are found in the nucleus and have even been shown to be involved in the transcriptional process (Pederson and Aebi, 2005; Percipalle and Visa, 2006). Although the kinetics of GFP-actin molecules in the nucleus could suggest the formation of polymeric nuclear actin (McDonald *et al*, 2006), the existence of a classical filamentous transport mechanism in the nucleus has yet to be demonstrated. On the other hand, a number of different approaches have shown that in fact the movement of mRNA in the nucleus is diffusion based. The movement of the total poly(A) mRNA population in the cell nucleus has been measured using the technique of fluorescent *in vivo* hybridization (FIVH). In this method, developed on the basis of the known fluorescent *in situ* hybridization (FISH) method applied to fixed cells (Levsky and Singer, 2003a; Shav-Tal *et al*, 2004b), either fluorescent or caged-fluorescent oligo-dT (or dU) probes were introduced into living cells thereby binding to poly(A) tails of all mRNAs. Following the movements of hybridized populations of mRNA-probe by either FRAP, FLIP, photoactivation or fluorescence correlation spectroscopy (FCS) showed that the intranuclear movement of mRNA was diffusion-based (Politz *et al*, 1998, 1999; Molenaar *et al*, 2004). Similarly, the movement of ribosomal RNA (rRNA) within the nucleoplasm followed the same biophysical rules (Politz *et al*, 2003).

The mobility analysis of nuclear mRNA has been approached also using protein tags based on GFP fusions. For instance, GFP-poly(A) binding protein 2 (GFP-PABP2) and the GFP-TBP export factor that bind mRNA were used in FRAP experiments for measurements of poly(A) mRNA diffusion coefficients (Calapez *et al*, 2002). As with the FIVH experiments described above, the advantage of mRNA tagging with RNA-binding proteins is the ability to study endogenous mRNAs. Poly(A) mRNA has been detected in nuclear speckles; however, live cell studies have repeatedly shown that the vast majority of mRNA moves freely through these domains and does not tend to accumulate or pause within them and probably does not serve as scaffolding for these structures (Politz *et al*, 1999; Molenaar *et al*, 2004; Shav-Tal *et al*, 2004a; Ritland Politz *et al*, 2006). The kinetic analysis of the mobility of a splicing factor (SF2/ASF-GFP), poly(A)-binding protein 2 (PABP2-GFP), and export factors (Aly-GFP, Tap-GFP) within the nucleoplasm and speckles has demonstrated that most of the nuclear pool of these proteins is not bound to mRNA, and is therefore available for the binding to newly synthesized transcripts (Molenaar *et al*, 2004). Interestingly, a recent study using the bimolecular fluorescence complementation (BiFC) assay (Hu and Kerppola, 2003), which allows the study of *in vivo* transient interactions between complexed molecules, has shown that the splicing factor Y14 and the nuclear export factor 1 (NXF1) interact with each other, and that these mRNA-Y14-NXF1 trapped complexes accumulate within and around speckles (Schmidt *et al*, 2006). Analysis of the dynamics of these complexes in speckles showed that about half are immobile thereby implying a function for speckles in mRNA export or in nuclear retention.

In order to overcome limitations in signal intensity detection and ability to tag specific transcripts, a unique RNA tag has been developed for the study of the dynamics of single RNA molecules. A unique sequence (MS2 sequence) originating from bacteriophage can form a stem–loop binding site in RNA, which is specifically bound by a phage capsid protein termed MS2 protein. Multiple repeats of the MS2 sequence are inserted into the DNA sequence of the gene under study, yielding an RNA molecule with multiple binding sites for MS2 proteins, which bind as dimers to each stem–loop. The expression of fluorescently-tagged MS2 (e.g. GFP-MS2) in cells, in conjunction with the expression of a gene containing multiple repeats of the MS2 sequence, provides a powerful system for the detection of single mRNP complexes above the diffuse nuclear GFP-MS2 background (Bertrand *et al*, 1998). Using rapid time-lapse imaging of fluorescently labeled single mRNP complexes, the real-time diffusion of individual nuclear mRNPs in living cells was tracked (Shav-Tal *et al*, 2004a; see Figure 2 and Supplementary Movie 3). While single particle tracking detected diffusive and corralled movements, the latter indicative of physical obstruction of mRNP movement by the chromatin environment, direct vectorial translocation of mRNPs as seen in the cytoplasm (Fusco *et al*, 2003), was never observed. Using the above systems it will be able to probe RNA expression on the single molecule and single gene level. FCS is already an available tool for studying gene expression at the single molecule level, and this technique will be able to resolve kinetics at specific points in the nucleus, for example, transcription sites or nuclear bodies. The issue of stochasticity versus timely ordered events in gene expression is also of main interest for *in vivo* studies. Stochastic expression seems to be the preferred model for activation of gene expression as seen from RNA FISH studies

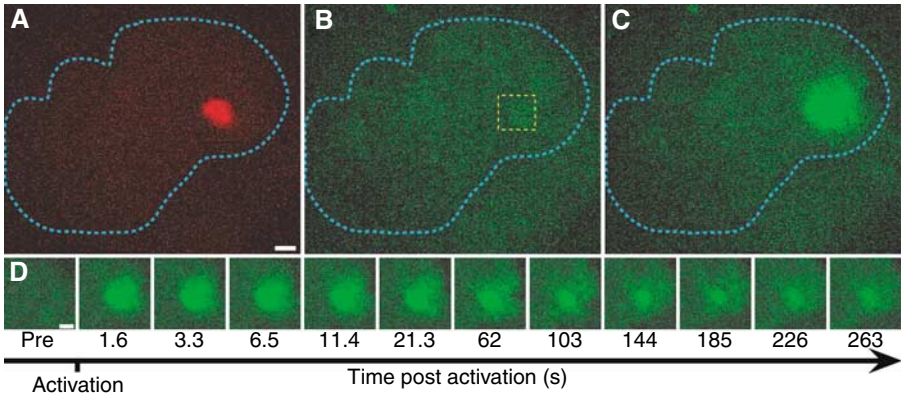

Figure 2 Measuring RNA movement by photoactivation. A DNA locus (detected in red by transfection of an RFP-lac repressor protein) that transcribes a tagged mRNA was co-transfected with photoactivatable MS2-GFP (MS2-paGFP) in order to fluorescently tag the mRNA. Transcription from this gene was induced for 30 min by doxycycline. The locus was detected (red) prior to photoactivation (**A**), and the image in GFP before activation was recorded (**B**). The 405-nm laser was directed at the boxed region of interest (yellow), and the MS2-paGFP was detected at the transcription site 1.635 s after activation (**C**). Bar, 2 μm. (**D**) The RNA signal emanating from the transcription site was followed for 262 s (bar, 2 μm). Adapted and reprinted from by permission from the American Association for the Advancement of Science: *Science* (Shav-Tal *et al*, 2004a), copyright 2004.

of endogenous gene loci performed in fixed cells (Gribnau *et al*, 1998; Levsky and Singer, 2003b). We perceive that the MS2 tagging system will yield a more global look at gene expression *in vivo* and efforts to this end have already proven that single gene expression dynamics can be resolved even for single endogenous genes (unpublished observations).

The dynamic nucleoplasmic landscape

RNPs traveling in the nucleoplasm are thought to move through a reticular network lying in between chromatin regions (Cremer and Cremer, 2001; Bridger *et al*, 2005). Yet, the nuclear interior also includes a number of unique compartments harboring specialized functions (Spector, 2001). These nuclear bodies self-assemble by virtue of nucleation around certain molecular components and are continuous with the nucleoplasm in which they reside, and in many cases their appearance and their numbers within the nuclear landscape are connected to cellular activity. Studies on the dynamic properties of the various nuclear domains have led to several major concepts that shape our understanding of nuclear organization.

Rapid exchange of nuclear body components

Nuclear domains were studied for many years in fixed cells using electron microscopy for fine structural characterization and later with fluorescently labeled antibodies using fluorescence microscopy. These studies established a view of a nucleoplasm containing well-defined and even rigid nuclear domains. Live-cell studies have modified this outlook. Notably, a rapid exchange of protein components between nuclear domains and the nucleoplasm has been identified, implying that the structural composition of these domains results of a steady-state flux of nuclear proteins. For instance, the most prominent nuclear domain—the nucleolus—whose gross structure is readily detectable under light microscopy is extremely dynamic and none of its components have been reported to be a permanent fixed component. The extreme structural scenario is the structural disappearance of this organelle during mitosis, although Nucleolar Organization Regions (NORs) contain RNA pol I and other protein associated with rDNA. Even during interphase, nucleolar proteins have been shown to exchange between the nucleolus and the surrounding nucleoplasm (Phair and Misteli, 2000; Snaar *et al*, 2000; Chen and Huang, 2001; Dundr *et al*, 2004; Louvet *et al*, 2005). rDNA is dynamic too (Roussel *et al*, 1996; Chubb *et al*, 2002), and the transcriptional state of the cell affects nucleolar structure and dynamics and even the position of some of the rDNA is affected by the transcriptional state of the cell (Angelier *et al*, 2005; Shav-Tal *et al*, 2005; reviewed in Hernandez-Verdun, 2006). The nucleolus also plays an important role in gene expression by acting as a domain in which many cellular regulators are sequestered, thereby modulating their cellular activity (Handwerger and Gall, 2006). A proteomic analysis of this organelle revealed that among the nearly 700 proteins present in the nucleolus, only a third are involved in rRNA biogenesis while the others were either unidentified or implicated in mechanisms known to take place outside the nucleolus (Andersen *et al*, 2005; Lam *et al*, 2005).

Another classical example for rapid mobility of proteins within the nucleoplasm is the nuclear speckles, also termed interchromatin granules or SC35 domains. These are enriched in factors involved in mRNA metabolism (Carter *et al*, 1993; Lamond and Spector, 2003). Speckles are probably not the sites of pre-mRNA splicing *per se* but might serve as a pool of stored or cycled factors destined to translocate and act on active nucleoplasmic genes (Singer and Green, 1997). Live-cell imaging of GFP-tagged splicing factors has shown that speckles are dynamic structures, whose structure is dependent on the activity levels of RNA polymerase II. Such studies have detected the budding off of small structures that might be indicative of transport of splicing factors from speckles to active genes (Misteli *et al*, 1997). FRAP of a GFP-tagged version of the splicing factor SF2/ASF showed similar recovery rates (~30 s) both in the nucleoplasm and in speckles, although in speckles an immobile population of less than 10% was detected (Kruhlak *et al*, 2000; Phair and Misteli, 2000). High mobility and short residence times (~50 s) within speckles were confirmed using kinetic modeling of the flux of SF2/ASF molecules between the nucleoplasm and speckles (Phair and Misteli, 2000). Single

molecule analysis of speckle-associated splicing factor U1 snRNP fluorescently tagged with Alexa488 or Cy5 showed that the protein is predominantly associated with speckles and is highly dynamic (Kues *et al*, 2001).

Since we have discussed the nucleolus and speckles, it is of interest to note that live-cell imaging has detected dynamic interconnections between the two domains. As with the nucleolus, during mitosis speckles disperse, once again implying structural assembly in interphase cells as a consequence of cellular activity. Moreover, live-cell experiments demonstrated that during telophase the SR splicing factors YFP-SF2/ASF and SC35-CFP first localized around active nucleolar organizing regions (NORs), and only later in G1, did they enter speckles (Bubulya *et al*, 2004; see Supplementary Movie 4). On the other hand, snRNPs were found together with SR proteins during telophase. Why splicing factors first assemble in the post-mitotic nucleolus remains to be determined, yet indications from transcriptional inactive cells show that there is cross-talk between splicing factors and the nucleolus that might include the binding of splicing factors to rRNA (Shav-Tal *et al*, 2005).

Nuclear roaming and relationship to gene loci

A second insight into nuclear dynamics is that most nuclear bodies seem to have the ability to roam through the nucleus and might be specifically associated with certain genes. The nucleolus, however, has fixed nuclear positioning dependant on assembly at specific chromosome regions.

Cajal bodies (CBs) can be seen in the nucleus of a cell by simple transmitted light (Cajal, 1903) and were purified (Lam *et al*, 2002), although their nature is still elusive. CBs move throughout the nucleoplasm. It was reported that their motion obeys diffusion rules, that they are occasionally corralled by chromatin domains and that interactions with chromatin depend on ATP (Boudonck *et al*, 1999; Platani *et al*, 2000; Platani *et al*, 2002; see Supplementary Movie 5). To date, the only catalytic function of CBs that has been demonstrated is the post-transcriptional modification of spliceosomal U snRNAs (Jady *et al*, 2003) that is mediated by a family of guide RNAs accumulating in CBs and that seem to be the best unique marker of this organelle (Darzacq *et al*, 2002; Liu *et al*, 2006). The U3 snoRNA transcription unit was described to associate in close vicinity with CBs (Gao *et al*, 1997). We recently found that an artificial gene array locus encoding an H/ACA box snoRNA was able to recruit CBs (Darzacq *et al*, 2006). Similarly, U1 and U2 snRNAs gene loci have been also reported to associate with CBs (Smith *et al*, 1995) and simultaneous detection of CBs, U2 gene DNA and U2 nascent transcript demonstrated the relation in between this association and the newly transcribed RNA being directly exchanged from its transcription site and the CB (see Figure 3; Smith and Lawrence, 2000). CBs are also the site of accumulation of the U7 snRNA involved in S phase expressed histone mRNA 3′ end processing (Strub *et al*, 1984; Bond *et al*, 1991) and histone gene loci were found to associate in close vicinity with the CBs, although their direct association with transcription of these genes is not clear (Frey and Matera, 1995; Shopland *et al*, 2001; Marzluff, 2005). The dynamic findings suggest that even if in close relation to specific genes loci, CBs are not linked to these genes but rather are loosely recruited or form *de novo* at locations where local concentrations of their substrates are found.

The PML protein, involved in an oncogenic translocation in acute promyelocytic leukemia, has been the defining protein of PML bodies. To date, numerous proteins have been shown to accumulate or pass through this body and many possible functions have been attributed to it. From a dynamic aspect, different types of movement were described for PML bodies using a YFP fusion to the Sp100 component of the PML body. These movements ranged from stationary to localized movement, and included also long-range movements. Interestingly, long-range movements were shown to be energy dependent (Muratani *et al*, 2002), while typically PML body movement as well as CB movement is diffuse or confined by the chromatin mesh in which these bodies can move (Gorisch *et al*, 2004). PML body distribution is sensitive to stress and under such conditions PML microstructures form due to budding off of the 'parental' bodies (Eskiw *et al*, 2003). After release from stress, there is fusing back of these microstructures to predefined locations indicating that PML bodies, which are typically stationary, are preferentially located at specific pre-determined locations that might be connected to certain genes (Shiels *et al*, 2001). PML bodies can be located in proximity to active gene regions although are not necessarily crucial for transcription *per se* (Eskiw *et al*, 2004; Wang *et al*, 2004). Furthermore, associations of PML with chromatin fibers are seen during mitosis (Dellaire *et al*, 2006a, b), and might imply a role for PML bodies in maintenance of genomic stability.

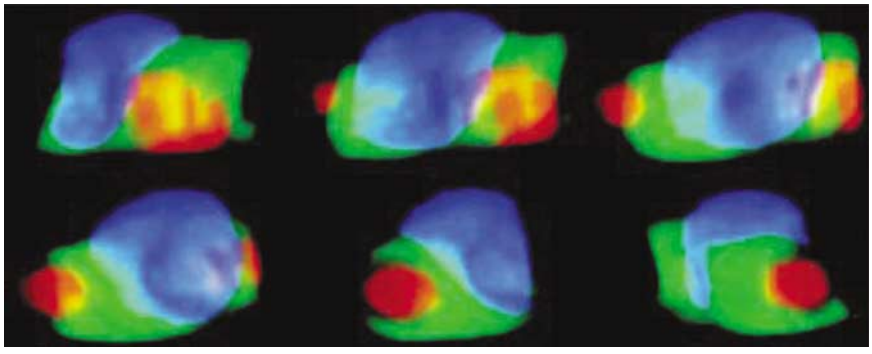

Figure 3 Three-dimensional visualization of CB, U2 gene locus and RNA. CB (blue) associated with two U2 loci (green) and RNA from the U2 locus (red). The close association of the CB and the U2 gene locus is evident, whereas the RNA foci do not appear to be as closely associated with the CB. Adapted and reprinted by permission from the American Association for Cell Biology: *Molecular Biology of the Cell* (Smith and Lawrence, 2000), copyright 2000.

In vivo transcriptional kinetics

The view of structurally fixed nuclear domains has disintegrated with the new dynamic information at hand. Similarly, thinking of transcriptional gene expression we tend to imagine relatively 'rigid' interactions of factor A with factor B to form an A + B complex that situates on the DNA and either induces transcription or is the transcriptional machinery itself. However, in light of the above, some kinetic flexibility must be introduced into our imaginary diagrams. For example, studies on the dynamics of transcription factors on promoter regions have introduced a 'hit and run' model in which rapid binding and release interactions of these factors on the DNA are observed (McNally *et al*, 2000; Muller *et al*, 2001; Stenoien *et al*, 2001a, b; Rayasam *et al*, 2005). This means that transcription activation or regulation is a net outcome of many dynamic assembly and disassembly events that stochastically lead to a favorable active complex. The recruitment of mRNA splicing and processing factors to an activated array of genes has been demonstrated *in vivo* (Janicki *et al*, 2004) and it remains to be seen whether these factors, which are part of spliceosomal complexes that are situated co-transcriptionally on nascent mRNAs (Neugebauer, 2002), also exchange rapidly with the nucleoplasmic pool.

Highly dynamic transcription factors regulate the transcriptional activity of RNA pol II. Recruitment times for GFP-Pol II to an integrated MMTV array were found to vary between single cells within a population, although transcriptional activation tended to peak at 20–30 min after induction (Becker *et al*, 2002). This might reflect the stochastic nature of the assembly of the transcriptional machinery on genes prior to activation. FRAP analysis of GFP-Pol II throughout the whole nucleus has allowed the identification of a number of polymerase populations: large complexes involved in active transcription versus freely diffusing unassembled subunits of GFP-Pol II, and also a population engaged in initiation events (Kimura *et al*, 2002; Hieda *et al*, 2005). The future analysis of GFP-Pol II on specific genes in the nucleus should yield important insights on the *in vivo* rates of mRNA transcription. As the nucleolus is the massive production site for rRNAs required for ribosome assembly, it was interesting to determine the *in vivo* kinetics of this transcriptional process. RNA polymerase I, which is the cellular polymerase exclusively responsible for transcribing rDNA, was analyzed in live cells by applying photobleaching procedures on GFP fusion proteins representing the major nucleolar constituents. This analysis was based on the principle that RNA polymerase I is only transcribing the 13.3 kb long rDNA transcription units in the nucleolus, and demonstrated that this activity was occurring at 95 bases per second (Dundr *et al*, 2002). Given the number of engaged polymerases observed in Miller spreads ($\approx$100) (Miller and Bakken, 1972), this would imply that an active rDNA gene could have a nominal activity of 1.4 rRNAs per second, meaning that as few as 107 rDNA active genes (out of 400 rDNA genes) could produce the rRNAs required for a cell. It therefore seems that even in an exponentially growing HeLa cell, the production of ribosomes is four times lower than the maximal calculated speed. One of the possible explanations of the apparent overproduction of rRNA by the pol I machinery could be the efficiency of rRNA maturation. Assembly of the different rRNAs with the nearly 80 proteins of the mature ribosome is a process that takes place mainly in the nucleolus and involves hundreds of small RNAs and polypeptides playing chaperone and control functions. It is still unclear how efficient this process is and the work described above suggests that half of the synthesized rRNA of a cell could be degraded before maturation.

Perspectives: from single cells to living organisms

The detailed view of gene expression dynamics arises from experiments typically performed in single living cells grown in tissue culture plates. Obviously, an important step in understanding the flow of gene expression is to study the dynamics within the context of the whole organism (Singer *et al*, 2005). This is a vital question since we do not yet know whether the dynamic behaviors we observe in tissue culture cells are of the same characteristics within tissues and living organisms, in which different types of cells are adjacent to each other and might be exchanging cell-to-cell signals. For example in bacterial cells, which do not have a nucleus, the dynamics of RNAs followed with an MS2-GFP tag exhibited Brownian motion, as seen in eukaryotes. Some RNA molecules remained tethered to the DNA while others diffused in the cytoplasm (Golding and Cox, 2004). This work served as the basis for the analysis of gene expression kinetics in single bacteria that showed that transcription in *Escherichia coli* occurs in quantal bursts (Golding *et al*, 2005). The implementation of the above-described methods for the study in whole organisms is wanting. The nucleus is useful for high-resolution imaging due to attributes of large size and rather uniform shape. We are probably still some steps away from following single RNA molecules in living organisms, but initial attempts in imaging of living organisms focusing mainly on the use of GFP-histone fusions have enhanced our understanding of chromatin dynamics in cell populations. This theme will increase with time with the development of imaging techniques, but following are some summarized examples of what potential live-cell imaging holds within living organism systems.

Multinucleated cells: fungi

The multinucleated features of the fungus hypha allow probing of questions related to the coordination of many nuclei in one cytoplasm. We described the movement of nuclear domains with the nucleus of mammalian cells, but following is an example of moving nuclei in a multinucleated organism. Live-cell imaging of H1-GFP histone fusion protein in multinucleated hyphae of *Neurospora crassa* has shown that nuclei are mobile and move along microtubules (Freitag *et al*, 2004; see Supplementary Movie 6). Interestingly, moving nuclei were pear-shaped and their leading edge contained a bright locus of H1-GFP, probably the area of attachment of the nucleus to the microtubule via a dynein motor. In another study of the multinucleated hyphae of *Ashbya gossypii*, it was shown that different nuclei within the hyphae can be cell-cycle independent of each other (Gladfelter *et al*, 2006). GFP-H4 (*AgHHF1-GFP)* labeled nuclei exhibited asynchronous mitotic divisions even though these nuclei were contained in the same cytoplasm. Cyclin proteins were abundant in the nuclei, their levels did not oscillate and therefore did not

appear to control the cell cycle. Experimental data from this system suggested that cell cycle in *A. gossypii* is controlled by cyclin-dependent kinase activity rather than by cyclins. Multinucleated cells are found also in mammalian tissues but we are still far from understanding the coexistence of such nuclei in one cell.

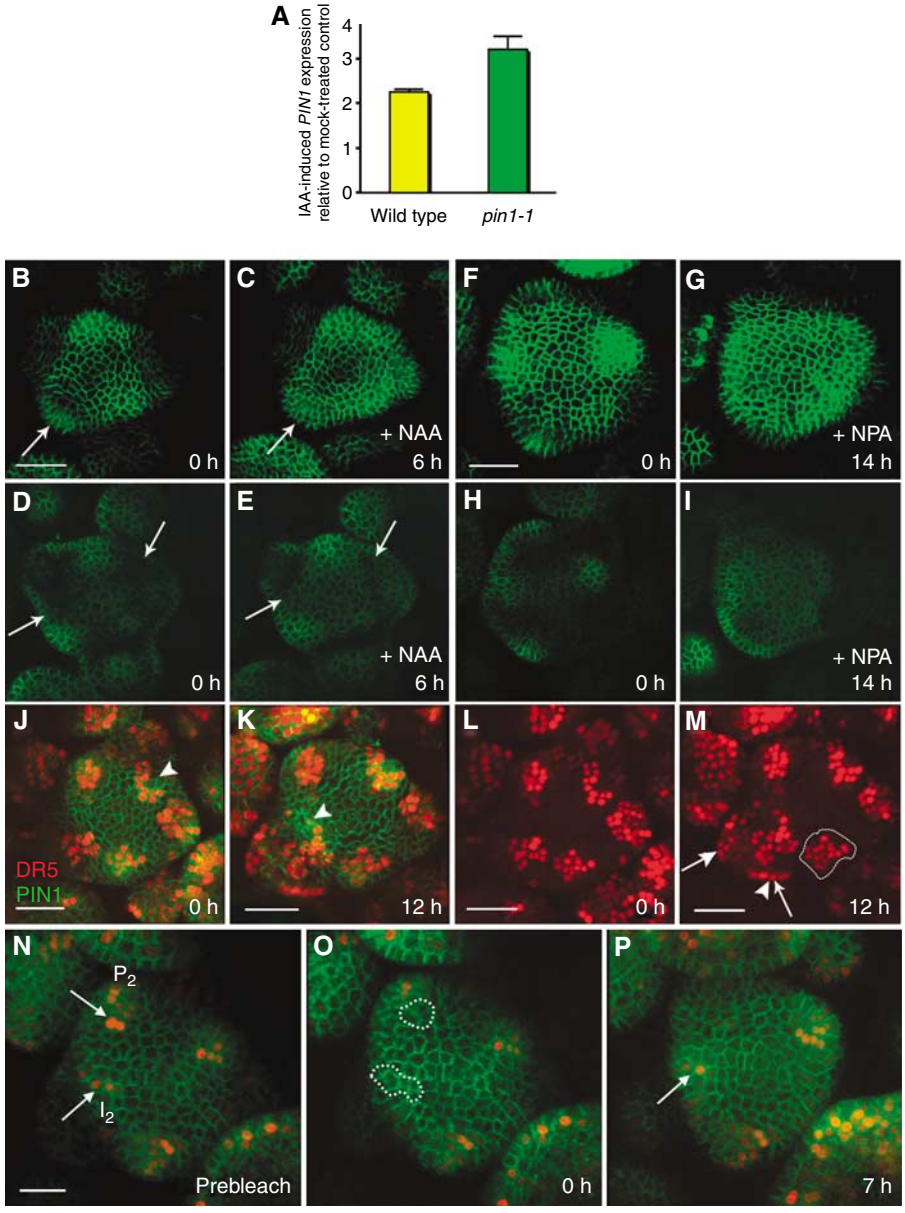

Figure 4 Monitoring gene expression during flower development in *Arabidopsis thaliana*. Confocal imaging of green fluorescentprotein (GFP) reporter genes was used in living plants to monitor the expression patterns of multiple proteins and genes involved in flower primordial developmental processes. The expression and polarity of PINFORMED1 (PIN1), the auxin efflux facilitator, was followed. (**A**) *PIN1* mRNA levels as measured by real-time PCR analysis of dissected wild-type inflorescences immersed in 100 μM IAA show greater than two-fold upregulation after 60 min relative to mock-treated controls. Identical treatments carried out on *pin1-1* mutant apices using 5 mM IAA in lanolin paste resulted in approximately three-fold induction after 30 min. (**B, C, F, G**) show maximum intensity projections of the meristem viewed from above, while (**D, E, H, I**) show corresponding transverse optical sections below the epidermis, respectively. (B–E) Response of *pPIN1::PIN1-GFP* (green) to exogenous auxin. (B, D) *pPIN1::PIN1-GFP*-expressing meristem before NAA treatment. (C) and (E) show the same meristem as in (B) and (D) after treatment with 5 mM NAA for 6 h. Expression becomes delocalized and increases in cells that previously expressed *pPIN1::PIN1-GFP* at low levels (arrows in (B–E)). This occurs both in the epidermis (compare (B) and (C)) and layers below (compare (D) and (E)). (F–I) Response of *pPIN1::PIN1-GFP* to treatment with 100 μM NPA for 14 h. In both the epidermis (F, G) and subepidermal layers (H, I), there is a delocalization of expression after 14 h. (**J–M**) Time lapse of *pDR5rev::3XVENUS-N7* (red) and *pPIN1::PIN1-GFP* (green) expression together (J, K) and *pDR5rev::3XVENUS-N7* (red) alone (L, M). At both the initial time point (J, L) and 12 h later (K, M), *pDR5rev::3XVENUS-N7* expression initiates when *pPIN1::PIN1-GFP* expression first marks a new site (arrowheads in (J) and (K)). After *PIN1-GFP* reverses polarity in cells adaxial to primordia, primordial expression of *pDR5rev::3XVENUS-N7* persists and subsequently appears in daughter cells of earlier-expressing cells (encircled by broken line in (M)). Expression in nondaughter cells occurs at a later stage of floral bud development (arrowheads in (M)). (**N, O**) Recovery of fluorescence after bleaching. Cells located within incipient primordia (I2 in (N)) and more mature primordia (P2 in (N)) that expressed *pDR5rev::3XVENUS-N7* were selectively irradiated with 514 nm laser light until expression became undetectable (circled regions in (O)). Seven hours after bleaching, fluorescence could again be detected in the same cells at I2 (arrow in (**P**)) but not in P2. Scale bars in (B), (F), and (J–N), 30 μm. Adapted and reprinted by permission from Cell Press: *Current biology* (Heisler *et al*, 2005), copyright 2005.

Growth and differentiation: plants

Plants have become an emerging easily manipulatable system for study of nuclear structure during growth and differentiation in a whole organism. *Arabidopsis thaliana* has proven to be a useful system for studying nuclear dynamics in plants. Integrated *LacO* or *tetO* repeats have been used to follow chromatin dynamics in different cell types (Kato and Lam, 2003; Matzke *et al*, 2005). For example, guard cells and pavement cells are found in the epidermis of seedlings and exhibit diploidy and polyploidy, respectively. GFP-LacI tagging showed constrained diffusional movement in both types of cells although in pavement cells the area of movement was six times larger than in guard cells (Kato and Lam, 2003). Interestingly, although the nuclear volume of polyploid pavement cells is greater than that of diploid guard cells, the ratio of nuclear space per genome remains similar. The authors of this study suggest that the appearance of more free space in the nucleus of pavement cells might indicate a lower degree of chromatin organization due to a reduction in the concentration of chromatin binding proteins. Tagging of endogenous *A. thaliana* H2B with YFP has demonstrated the succession of nuclear divisions in living root tissues that lead to the development of a syncytium, and has found that this developmental process takes place independently in three specific mitotic domains within the root endosperm (Boisnard-Lorig *et al*, 2001). The patterns of cell division in growing shoot apical meristem were imaged in real-time and revealed heterogeneity in the division rates across different cell layers (Reddy *et al*, 2004). This system for plant development imaging was further implemented in the monitoring of expression of multiple proteins involved in the development of flower primordia (Heisler *et al*, 2005; see Figure 4). In plants there is only one form of heterochromatin binding protein 1 (HP1), while other species have several forms that localize to different chromatin regions. GFP-tagged plant HP1 (LHP1) localized to heterochromatic chromocenters and to speckle-like nucleoplasmic domains and FRAP analysis showed high mobility of this protein as observed in mammalian cells (Zemach *et al*, 2006).

Imaging of centromeres in living *A. thaliana* plants has shown radial positioning and constrained movement in the nuclear periphery of different cell types, suggesting anchoring of chromosomes. Centromere positioning was not transmitted through cell division, contrary to some observations in human cells (Fang and Spector, 2005). This might suggest that epigenetic information in plants is not necessarily encoded in the positioning of chromosomes in the nucleus. Nuclear speckles in plants were found to have similar characteristics as their mammalian counterparts, exhibiting constrained movement together with rapid exchange of protein components, together with budding off of speckles and structural sensitivity to transcriptional inhibition (Ali *et al*, 2003; Fang *et al*, 2004; Tillemans *et al*, 2005). Dynamic CBs were found both in the nucleoplasm and the nucleolus of plant cells, at times moving from the nuclear periphery into nucleoli (Boudonck *et al*, 1999). The similarities found between mammalian and plant gene expression systems encourage the implementation of *in vivo* bio-imaging techniques in plants and indeed sprouting studies that utilize techniques such as FRET-FLIM (Immink *et al*, 2002) and BiFC (Bracha-Drori *et al*, 2004) show potential in unraveling interactions between proteins in nuclei.

Developing embryos: Drosophila and zebrafish

As in other systems, chromosome dynamics have been studied in developing *Drosophila* embryos. Using the *lacO*/LacI system, long-range movements of chromatin were observed although these were cell-cycle stage-specific and tended to decrease and disappear during spermatocyte (Vazquez *et al*, 2001) and eye imaginal disc cell-differentiation (Thakar and Csink, 2005). The decrease in movement is probably due to events of nuclear compaction that accompany differentiation. In addition, as found with labeling of chromatin with a fluorescent topoisomerase protein, short-range constrained motion of Brownian nature is always detected (Marshall *et al*, 1997). Photoactivation of a paGFP-histone protein using two-photon microscopy also showed constrained motion in stage 5 embryos (Post *et al*, 2005).

Zebrafish embryos are transparent and therefore set the stage for live-cell imaging of developmental aspects of gene expression (Megason and Fraser, 2003). The orientation of mitotic divisions in developing zebrafish have been studied by injection of a construct encoding an H2B-GFP fusion protein to gastrulae (Gong *et al*, 2004) or embryos (Das *et al*, 2003). These studies showed that cells in dorsal tissues divide along the animal-vegetal axis of the developing embryo, and that in fish retina one of the daughter cells retains its physical connection with the basal surface of the retina after mitosis.

Concluding remarks

Real-time imaging in single cells and organisms has allowed gene expression to unravel before our eyes. No longer are components of chromatin, nucleosomes and the transcriptional machinery merely protein bands on a Western blot or fluorescent dots in a fixed cell, but can now be followed as they actively assemble and interchange at gene loci and sites of transcription. 'Seeing is believing' is not just a cliché, and with bio-imaging techniques we can now provide detailed time-resolved molecular information about protein assemblies in living cells. With such tools at hand, our efforts now proceed in two paths. On the one hand, we strive to reach the resolution of single genes and single molecules, and examine the kinetics of expression of single or endogenous genes. The latter are best studied either in primary cells or even better within the live organism. On the other, transgenic mice expressing GFP-tagged cells can be used for tracking of fluorescent cells within the animal (Hadjantonakis and Papaioannou, 2004; Fraser *et al*, 2005). We anticipate further developments of such mammalian systems for the study of gene expression in the context of the normal and tumorigenic living tissues.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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