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Drosophila under the lens: imaging from chromosomes to whole embryos

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Abstract

Microscopy has been a very powerful tool for *Drosophila* research since its inception, proving to be essential for the evaluation of mutant phenotypes, the understanding of cellular and tissue physiology, and the illumination of complex biological questions. In this article we review the breadth of this field, making note of some of the seminal papers. We expand on the use of microscopy to study questions related to gene locus and nuclear architecture, presenting new data using fluorescence *in-situ* hybridization techniques that demonstrate the flexibility of *Drosophila* chromosomes. Finally, we review the burgeoning use of fluorescence *in-vivo* imaging methods to yield quantitative information about cellular processes.

Introduction

Microscopy, a tool to dissect Drosophila

Drosophila research has relied on microscopy: (a) to delineate the myriad of mutants by eye color, bristles, bars or wing shape; (b) to determine lethal endpoints in development; and (c) to annotate the breakpoints of translocations by comparison with the long-used maps of Bridges (1935). The first *in-situ* hybridizations were carried out on polytene chromosomes (Spradling *et al.* 1975), reaffirming the correlation of genes to the linear sequence of polytene bands as established by early mapping (Painter 1934), and gene cloning soon allowed the localization of any gene sequence by these techniques (Wensink *et al.* 1974). P1 phage regional clones in the early 1980s and later YAC and BAC clones of much of the *Drosophila* genome became available for genome walking and fine mapping (http://flybase.bio.indiana.edu/) (Drysdale & Crosby 2005). In addition, they provided DNA probes for *in-situ* hybridization that have illuminated the mechanism of transvection and nuclear architecture (Fung *et al.* 1998, Gemkow *et al.* 1998); an area that we will treat in detail below and for which we present new data on homolog pairing.

Early in the age of molecular genetics by means of β-galactosidase fusions with genes and promoters, the *Drosophila* community was able to determine expression boundaries of genes and control elements in time and space. However, the great breakthrough was the wedding of fluorescence microscopy with this ideal meso/microscopic organism, possessing a

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transparent embryo, that opened up the possibility to correlate information about gene activity and localization with structure and developmental stage or mutant phenotype. We need only look at the multicolor *Drosophila* embryo displaying the gradients of gap and pair rule genes to appreciate the revolution and synergism that resulted from such a union (http://flyex.ams.sunysb.edu/FlyEx/ and composite Figure 1 derived from this site). Detailed information on ontogeny came from the application of fluorescence microscopy by Foe (1989) for the progression of cell replication and division throughout embryogenesis. However, it was not until *in-vivo* imaging was made possible by the introduction of fluorescently labeled proteins, first by microinjection into embryos (Kellogg *et al.* 1988) and later by the introduction of genes encoding fluorescent proteins, that lineage could be followed for many cell generations and the migration of cells specified in detail. A limited lineage exposition was demonstrated with microinjected, labeled histones and time-lapse microscopy by Minden *et al.* (1989). Such studies were expanded first with *in-vivo* labeling of cells by lipid probes (Bossing & Technau 1994) and then with green fluorescent protein (GFP) markers using tissue-specific promoters with which the developmental progression of complete organ systems in embryos, larvae and oocytes could be traced (Schmid *et al.* 1999). *In-vivo* imaging is not limited to lineage studies but encompasses all manner of functional assays in wild-type and mutant *Drosophila*. For example, the early technique of histone labeling of embryos allowed our laboratory to determine the *in-vivo* mechanism of topoisomerase II inhibition by drugs such as VM26 compared with inhibitor antibodies (Buchenau *et al.* 1993).

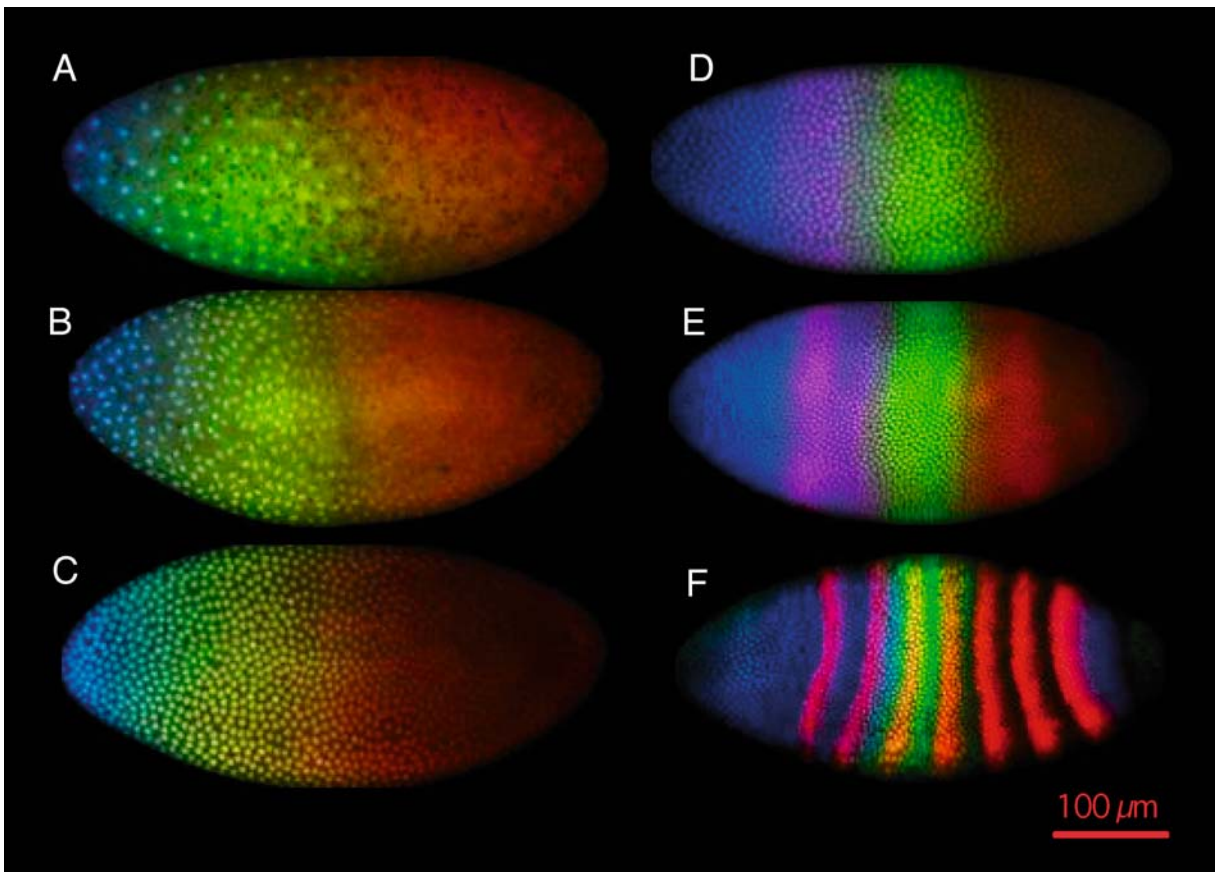


Figure 1. Expression patterns of gap and pair-rule genes in *Drosophila* embryos. **A**–**C**: red, even-skipped; green, hunchback; blue, bicoid; **D**–**F**: red, even-skipped; green, Kruppel; blue, hunchback. **A**, cycle 10; **B**, cycle 12, **C** and **D**, cycle 13; **E**, cycle 14/2; **F**, cycle 14/4. Anterior to the left, dorsal to the top. Images used by permission (Kosman *et al.* 1998, 1999) from *http://flyex.ams.sunysb.edu/FlyEx/.*

More recently, high-resolution fluorescence microscopy has been employed to observe *in vivo* the movement of macromolecules within cells, tissues or whole embryos, e.g. by using molecular beacons to track mRNA (Bratu *et al.* 2003), or by using fluorescence recovery after photobleaching (FRAP) techniques to study GFP fusions of chromatin protein complexes (Ficz *et al.* 2005), as will be discussed below.

Finally, two techniques have been introduced for the *in-vivo* study of chromatin mobility and nuclear architecture in *Drosophila*: (a) the integration of bacterial DNA operator sequences into the *Drosophila* genome that can bind bacterial repressor proteins fused to GFP, and (b) the expression of photoactivatable GFP fused to histones. These techniques and the results achieved to date are reviewed in the final part of this article.

FISH in whole mount embryos and imaginal discs

Fluorescence *in-situ* hybridization (FISH) and high-resolution confocal microscopy have provided detailed insights into gene locations and snapshots of the behavior of chromosomal loci throughout development. Using these techniques specific distances between loci have been measured with the precision afforded by light microscopy and have allowed one to test directly theories concerning transvection and chromosome mobility.

Flexibility of interphase chromosomes

Csink & Henikoff (1998) described large-scale chromosomal movements, particularly in S-phase, by analyzing a FISH probe within the 2R centromeric heterochromatin and one at the telomere of 2R. In earlier studies they demonstrated that Heterochromatin Protein 1 (HP1) promotes the association of distant heterochromatic loci and their subsequent silencing (Csink & Henikoff 1996, Dernburg *et al.* 1996). Sigrist & Pirrotta (1997) observed the consequences of the *trans* interaction of different Polycomb Response Elements (PREs) inserted on homologous chromosomes that resulted in the suppression of reporter gene transcription. They inferred from their phenotypes that the same PRE inserted on heterologous chromosomes can also interact. Their data are consistent with direct observations by FISH: (a) by Gemkow *et al.* (1998) showing interaction of translocated elements containing PREs on heterologous chromosomes and (b) more recently by Bantignies *et al.* (2003), who looked by FISH at the homing effect of the *Fab-7* locus. In this latter study a segment containing the strong *Fab-7* PRE preferentially pairs with the endogenous *Fab-7* locus when inserted as a transgene (see also Grimaud *et al.* 2006).

The fact that homolog chromosome pairing begins at the mid-blastula transition in embryonic development was definitively demonstrated in two laboratories by FISH studies (Fung *et al.* 1998, Gemkow *et al.* 1998). The frequency of pairing was dependent on the length of the interphase, reaching a maximum of ~80% in post-mitotic embryonic nuclei. Gemkow *et al.* (1998) showed that transvection, the phenomenon by which genes are regulated *in trans*, is most likely promoted by direct pairing and/or looping of the DNA strands from homologous chromosomes. Further evidence for the flexibility of *Drosophila* chromosomes was obtained in this study by the fact that translocations of a segment containing one copy of the *bithorax* gene cluster (*BX-C*) from chromosome 3R to the opposite arm (3L) or to the X chromosome eliminated transvection, but did not abrogate homolog pairing. We were interested as to whether this long-distance pairing was compatible with normal homolog pairing in the adjacent sequences. In the following section we present data to answer this question.

We used *in-situ* hybridization data to compare the pairing frequency of a locus within a translocation segment as well as one near the breakpoint of the segment excised from chromosome 3R to test the flexibility of *Drosophila* chromatin. The frequency of a P1 *Drosophila* DNA probe located inside the translocated segment and proximal to the *BX-C* in female *Tp(3;1) P115/w;Df/+* flies (Figure 2A), and those for a probe lying immediately distal to the

Table 1. Pairing frequencies for DNA probes inside (*DS08009*) or outside (*DS04079*) the P115 translocation from 3R to X

Probe	Unpaired loci	Paired loci	Pairing frequency
DS08009	235	143	38% ± 4%
DS04079	167	372	69% ± 10%

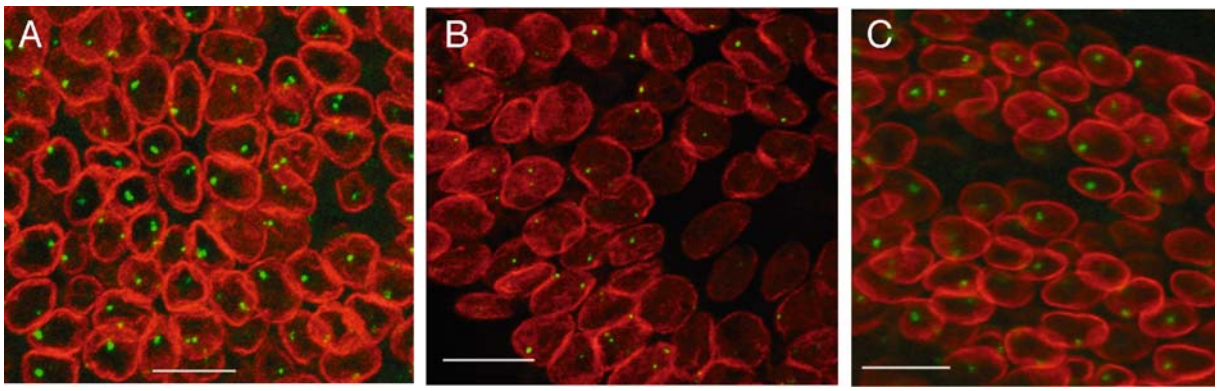


Figure 2. Views of FISH signals in whole mount imaginal disc nuclei. **A**: Wing disc from translocation *Tp(3;1) P115/w;Df/+*flies hybridized with a probe inside the translocated segment. **B**: Eye disc from $su(Hw)^{-/-}$ flies hybridized with a probe in the band 89B11. **C**: Wing disc from $su(Hw)^{-/-}$ flies hybridized with a probe to the histone gene locus. Red, anti-lamin Mab T40, Cy5; green, Cy3 anti-digoxigenin. Images are extended views of three *z* image planes after background subtraction and contrast stretching (bar = 5 μm).

translocated segment on chromosome 3R in the same line, are given in Table 1. The pairing frequency was significantly higher for the segment located outside the loop (Student's *t*-test null hypothesis, 0.011) and similar to that found for a variety of loci on chromosomes 2 and 3 in previous studies, demonstrating that the translocation does not inhibit normal homolog pairing in adjacent segments. In addition, the mean pairing frequency of 37% found for the translocated segment was close to that measured for probes in the *Ultrabithorax (Ubx)* and *Abdominal-B (Abd-B)* genes (within the *BX-C*) in this segment (Gemkow *et al.* 1998) and represents a very significant interaction of segments from two non-homologous chromosomes, the 3R and X. The random association of non-homologous loci without PREs or heterochromatic elements between different chromosomes was less than 1% (data not shown). These data exclude that only the PRE sequences are promoting pairing in the translocated segment. These results are also consistent with the hypothesis that *Drosophila* chromosomes are highly flexible and search for homologous regions over a large part of the nuclear volume.

In contrast to these results from *Drosophila*, homologous pairing has been detected only in mammalian neuronal cells (Arnoldus *et al.* 1989, 1991) and not in mammalian tissue culture cells, where the only large-scale movement in interphase is dependent upon induction of a strong viral promoter (Tumbar *et al.* 1999) or a change in the state of differentiation (Brown *et al.* 1999, Volpi *et al.* 2000).

The influence of protein interactions on homologous pairing

Based on our observations of long-range homolog recognition, we proposed that chromosome pairing in *Drosophila* is promoted by a multipoint recognition along the chromosome, resulting in globally stable interactions despite relatively unstable (short lifetime) individual associations, and suggested that some of these protein–protein interactions responsible for recognition and pairing might be enhancer and/or repressor complexes (Gemkow *et al.* 1998). Gerasimova *et al.* (2000) suggested that proteins bound to insulator sequences organize the chromatin into domains by forming higher-order intra- and interchromosomal loops from FISH data. Suppressor of Hairy wing, Su(Hw), is a zinc finger protein that binds to an octomer repeat found in enhancer elements of various genes as well as to the retrotransposon *gypsy* insulator (Georgiev & Corces 1995, Geyer 1997, Chen & Corces 2001). Su(Hw) can act *in trans* and inactivate enhancers located on the homologous chromosome. This phenomenon is inhibited by chromosomal rearrangements that disrupt pairing, suggesting that close apposition between the two copies of the affected gene is important for *trans* repression of transcription. Thus, we chose to investigate the effect of a null mutation in the *su(Hw)* gene on homolog pairing in larval imaginal discs.

FISH was performed in wild-type and mutant imaginal discs and the frequency of homologous pairing calculated. The data for two P1 DNA probes

Table 2. Pairing frequencies in wild-type (WT) and *su(Hw)*$^{-/-}$ imaginal disc nuclei

Strain	Locus	Unpaired loci	Paired loci	Pairing frequency
su(Hw)$^{-/-}$	DS03126	115	228	65.5% ± 10.5%
WT	DS03126	22	660	97 ± 4.1%
su(Hw)$^{-/-}$	DS03648	230	481	68% ± 3.2%
WT	DS03648	52	564	92% ± 2.7%
su(Hw)$^{-/-}$	Histones	8	200	96.1% ± 2.8%
WT	Histones	13	655	98.2% ± 2%

su(Hw)$^{-/-}$: (y^2 *w scr*^{t6} *uf*; *su(Hw)v/TM6B* and y^2 + X *su(Hw)v*, *cas X/K/TM6B*).

that do not contain a *gypsy* binding site can be found in Table 2. Comparing the results for the mutant and wild-type strains shows that the pairing frequency decreases by ~30% in *su(Hw)*$^{-/-}$ homozygous embryos. A probe in the BX-C and one outside this region (Figure 2B) were affected similarly. These results imply that PcG complexes alone do not suffice for driving homolog recognition despite their strong effect on homing and long-distance pairing effects cited above.

We used the histone gene cluster as a control for these studies. It contains all of the major histones, including the intergenic sequences, in 100 copies arranged in tandem duplication at a single locus in *D. melanogaster* (Lifton *et al.* 1978). In embryogenesis the zygotic histone gene cluster is transcribed very early to provide histones to the rapidly dividing embryo. Fung *et al.* (1998) showed that this locus pairs before any other locus and reaches a higher pairing frequency than other tested sequences in *Drosophila* embryos. We found that the histone genes were consistently paired in both wild-type and mutant imaginal discs at a frequency of > 95% (see Table 2 and Figure 2C). The obvious dissociation of this locus from factors that drive recognition of non-repetitive euchromatin is consistent with the special nature of this DNA and its transcriptional properties that may provide an early nucleation site for pairing.

Figure 3 plots the normalized frequency of interhomolog distances between the unpaired homologous alleles in *su(Hw)* mutants compared to distances of unpaired loci in post mitotic wild-type nuclei and early embryonic wild-type nuclei. Early in embryogenesis at cycle 14 (stage 6), when only 10–20% of the homologs are paired, the distances between unpaired loci are distributed randomly over the whole nuclear volume, whereas in late G1 arrested embryos (> stage 10), when pairing reaches its maximum level, the interhomolog distances of the unpaired loci are restricted to small values (mean at ~ 1 μm) indicative of only local dissociation and restrained diffusion of the chromatin (see Gemkow *et al.* 1998, Figure 7, and the discussion in the penultimate section of this article). Although the pairing frequency was significantly decreased for loci other than the histone gene cluster, the interhomolog distances for unpaired loci in *su(Hw)* mutants were small (Figure 3), similar to the distribution of distances found in wild-type post-mitotic embryonic and larval nuclei. Earlier data on *zeste* mutants in embryos demonstrated local decondensation effects but little reduction in pairing frequencies (Gemkow *et al.* 1998). Taken together, these data imply that numerous different protein complexes along the chromosomes serve as recognition sites for pairing and that chromatin is in a dynamic equilibrium between pairing and non-pairing. Thus, in most cases, the removal of a single protein species that organizes higher-order complexes will affect local interactions but movements will be constrained by other complexes in adjacent segments.

Dynamics and live cell imaging

The cloning of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie *et al.* 1994) and its expression in eukaryotic cells has revolutionized developmental biology. Many GFPs and coral fluorescent protein derivatives, visible fluorescent proteins (VFPs), have been developed during the past 10 years, allowing the simultaneous observation of as many as six separable VFPs (Miyawaki 2006). Many of these mutants possess increased photostability as well as the property of photoconvertability between different fluorescent forms (Lippincott-Schwartz &

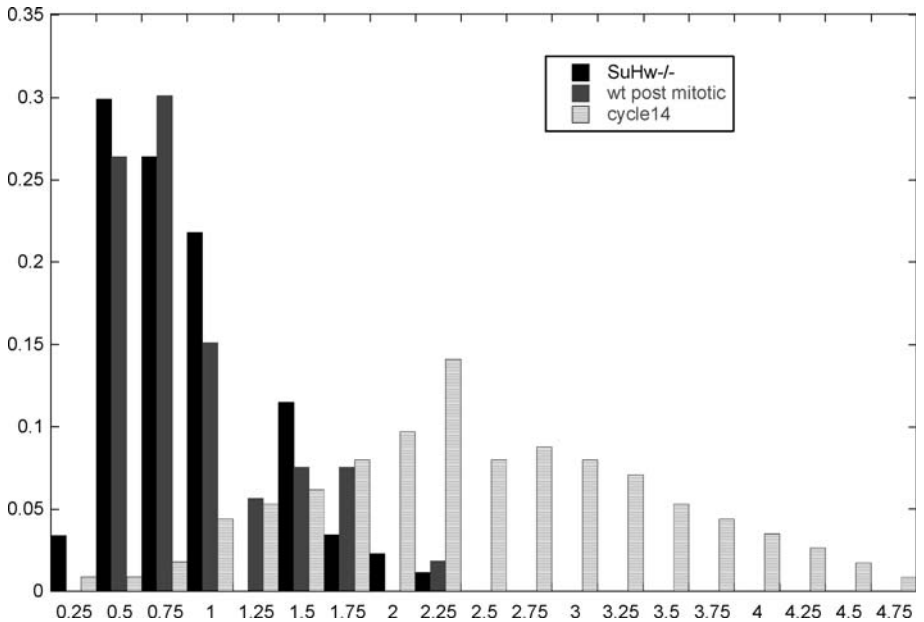


Figure 3. Relative frequency of unpaired interhomolog distances. Solid black bars, *su(Hw)*$^{-/-}$ nuclei; gray bars, wild-type post-mitotic nuclei; hatched bars, embryonic nuclei in early cycle 14. The abscissa is in microns.

Patterson 2003, Ando *et al.* 2004). In combination with confocal microscopy, VFPs provide enormous possibilities for labeling cells and whole organisms including the analysis of dynamic processes such as protein diffusion or chromatin movement *in vivo*. It is impossible to review the plethora of publications that have used VFPs in this area in the past 10 years, and we will therefore restrict our discussion to a few examples of applications in *Drosophila* from the labeling of whole organisms to the study of protein dynamics.

Whole animals and clones of cells – GFP marked chromosomes

One important feature that made *Drosophila* such a useful organism for genetic studies is the existence of balancer chromosomes. These special chromosomes contain chromosomal inversions that lead to loss of genes upon recombination with their homologous segments resulting in embryonic lethality, and thus provide a means to maintain mutants in heterologous crosses or lines. Balancers exist for chromosomes X, 2 and 3; the fourth *Drosophila* chromosome is very small, heterochromatic, and usually does not recombine. Balancer chromosomes carry dominant markers that help to identify them in adult flies. The addition of GFP as a marker for balancers has made it possible to identify flies that carry these balancers at much earlier developmental stages (Casso *et al.* 1999, Halfon *et al.* 2002). Starting in embryos at the extended germband stage GFP expression driven by the *Kruppel* promoter, for example, can be detected in all stages through embryonic and larval development as well as in pupae and adult flies. Homozygous mutant animals can be selected, due to the absence of the GFP marker even if they do not show a mutant phenotype at a given stage, and thus be subjected to gene expression microarray analysis (Furlong *et al.* 2001a,b).

GFP has also proved to be an indispensable tool to mark homozygous mutant clones of cells induced within a tissue by use of an FLP recombination target (FRT) sequence close to their centromeres that mediates mitotic recombination induced by the expression of FLP recombinase (Xu & Rubin 1993) in a certain tissue or under the control of a heatshock promoter. The homologous chromosome with an FRT site near the mutation of interest is recombined to produce a clone of homozygous mutant cells labeled by the absence of GFP within a green tissue. With this

method one can analyze alterations of gene expression in the mutant clones (Beuchle *et al.* 2001) or generate germline clones resulting in embryos that lack maternal gene products (Luschnig *et al.* 2004).

Specific cells, tissues and subcellular compartments

The GAL4/UAS enhancer trap technique (Brand & Perrimon 1993, Gerlitz *et al.* 2002) allows the expression of a reporter gene fused to the upstream activating sequence (UAS) from yeast to be expressed in the presence of Gal4. Random insertion of the reporter gene construct allows transcription under the control of endogenous enhancers with readout of the enhancer phenotype through expression patterns of the fusion protein (see Marr *et al.* 2006). Originally bacterial β-galactosidase was used as the reporter, but VFP readout affords certain advantages, such as: (a) no need for prior fixation or the addition of substrates or antibodies; (b) real-time observation of living embryos and larvae as well as cultured egg chambers; (c) high-resolution analysis using confocal laser scanning microscopy (CLSM) images (Yeh *et al.* 1995); (d) fluorescence-assisted cell sorter (FACS) selection of dissociated GFP-labeled tissues for expression array analysis (Amrein & Axel 1997); and (e) cell lineage analysis (Murray *et al.* 1998, Kraut & Zinn 2004).

The most obvious use of VFP fusion proteins has been to localize and quantitate proteins and subcellular compartments throughout development. Similarly they have been applied to questions of chromatin protein localization, e.g. Polycomb (Dietzel *et al.* 1999) and Cid, the *Drosophila* CENP-A homologue (Ahmad & Henikoff 2001).

Dynamics of individual chromosomal regions and loci associated with GFP fusion proteins

In a previous section of the article we presented evidence for chromosome mobility from FISH data that provide 'snapshots' in time of dynamic processes. The use of GFP probes attached to chromosomal loci or associated with tightly bound chromosomal proteins constitutes an *in-vivo* method for ascertaining chromosomal mobility directly. We have used transgenic *Drosophila* embryos, expressing an H2AvD-GFP fusion protein (Clarkson & Saint 1999, Saint & Clarkson 2000) to observe the distribution of the chromatin in living embryos as shown in the supplementary movie of real-time chromatin movement in a live cycle 13 embryo. One can discern chromatin associations all along the nuclear envelope as well as long-range chromatin movement over second and minute time scales, particularly in the interior of the nucleus. Such rapid movement is reminiscent of loci consisting of lac operator arrays

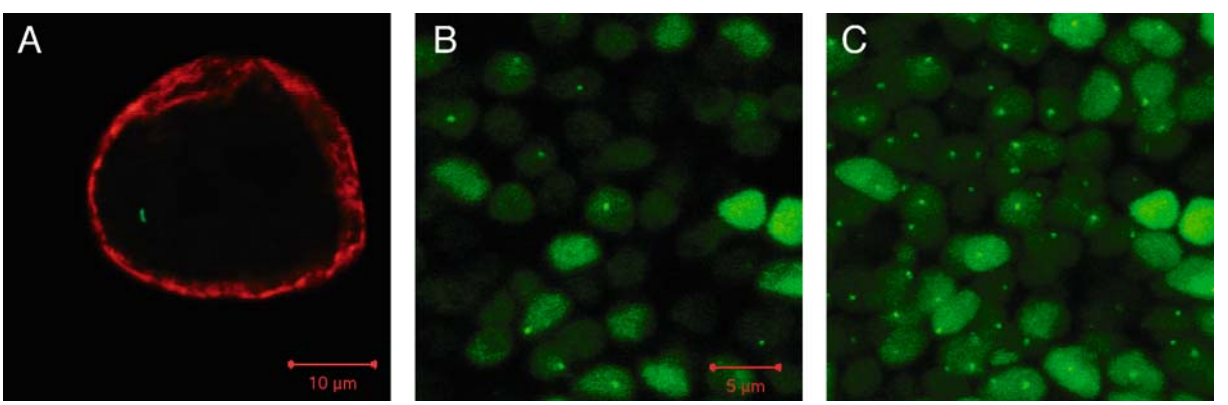


Figure 4. Binding of lac repressor to lacO arrays in chromatin allows tracking of individual sites. **A**: *lacO* array integrated into the third chromosome visualized by GFP-labeled lac repressor binding (green) in a salivary gland nucleus of a third-instar *Drosophila* larva. The integration site appears as a bright band on the polytene chromosome. Counterstaining of the nuclear lamina with mRFP-Lamin DmO. The expression of both fluorescently labeled proteins was induced by heatshocking the larvae 24 hours before dissection. **B**: Wing imaginal disc nuclei from the same transgenic line showing one single *lacO* array per nucleus. Unbound GFP-lac repressor stains the entire nuclear volume. Single *z*-section. **C**: Projection of the whole *z*-stack (15 × 0.75 µm). Same original magnification as in **B**.

in yeast nuclei (Heun *et al.* 2001a,b). Similar chromatin movement was observed in *Drosophila* larval imaginal discs using Pc-GFP or Ph-GFP fusion proteins (see Figure 3 and supplementary movie 1 in Ficz *et al.* 2005).

The marking of the entire genome by the variant histone H2AvD-GFP does not permit tracking of individual loci for quantitation of diffusion constants. However, the mutation of the GFP to a photoactivatable GFP (paGFP) (Post *et al.* 2005), followed by simultaneous two-photon activation of loci of 0.2–0.8 μm^2 in nuclei of embryos and larval explants, provided a means to perform 2-D tracking and to compare various nuclear locations at a variety of development stages (Post *et al*. manuscript in preparation).

In another method for tracking individual chromosomal loci, a GFP-labeled lac repressor is targeted to its high-affinity binding sequence, the *E. coli lactose operator* (*lacO*), integrated into eukaryotic chromatin (Robinett *et al.* 1996, Straight *et al.* 1996). The relatively high background of unbound lac repressor can be compensated for by the size of the *lacO* insertion or by modulating the expression levels of lac repressor protein to achieve a good locus signal to background. On the other hand, the background staining of unbound lac repressor can also be utilized to visualize the size and shape of the nuclei without additional staining, although nuclear membrane labeling by another VFP protein is to be desired for very accurate determination of the nuclear boundary (Heun *et al.* 2001a,b) (Figure 4A–C).

P-elements allow the efficient insertion of small *lacO* arrays in *Drosophila* without tandem duplication of the arrays with endogenous sequences such as occurs in mammalian genomes. The first application in *Drosophila* of the *lacO* system was for the analysis of chromatin movement in premeiotic nuclei of spermatocytes, a highly specialized tissue (Vazquez *et al.* 2001). In this tissue chromatin undergoes both short-range (fast) and long-range (slow) constrained random motion with a larger radius of gyration for the latter. Recently Thakar & Csink (2005) demonstrated that chromatin movement is more constrained in differentiated than in undifferentiated cells with the *lacO* system, presumably due to a general nuclear contraction and/or chromatin compaction as cells become smaller. The authors used *Drosophila* eye imaginal discs in which it was possible to analyze the undifferentiated cells in front of the morphogenetic furrow together with differentiated cells after the morphogenetic furrow in one and the same disc.

In an analogous approach to the *lacO*/GFP lac repressor system, Buchenau *et al.* (1997) injected a fluorescently labeled antibody against Hrb57A, a *Drosophila* homolog of hnRNP K proteins, into living *Drosophila* embryos. Upon heatshock the Hrb57A protein localizes mainly to the 93D locus,

Table 3. Comparison of published diffusion constants for chromatin

Nuclei	Reference	Diffusion coefficient D ($\times 10^{-3}$ μm^2/s)
Drosophila spermatocytes	Vazquez *et al.* 2001	
Early G2 short range, long range		13, 1
Late G2 short range, long range		3.4, 0.094
Drosophila eye disc	Thakar & Csink 2005	
Undifferentiated, differentiated		0.33, 0.21
Primary culture CNS cells		0.37
Drosophila embryos	Marshall *et al.* 1997	2.0
Drosophila embryos stage 5	Post *et al.* 2005	20
Yeast GFP-lacI		
Chromosomal	Hediger *et al.* 2004	1–10
Plasmid	Marshall *et al.* 1997	0.3
Neuroblastoma Cy3-dUTP labeled chromosomes	Bornfleth *et al.* 1999	
Free diffusion		0.05
Human HT-1080 with 128-mer lacO array 7–16 copies	Chubb *et al.* 2002	
5p14 locus		0.125
Chinese hamster ovary cells	Levi *et al.* 2005	
Slow diffusion, fast diffusion		0.24, 3.13

resulting in a bright spot that can be tracked. Individual loci showed different types of movements: jitter, slow movements, rapid movements over both large and small distances and, apparently, both random walk and directed movements. However, this approach allowed the tracking of only one specific chromatin locus under stress conditions. In the same year Marshall *et al.* reported the injection of fluorescently labeled antibody to topoisomerase II which shows a very large accumulation at the 359 bp satellite repeat sequence on the X chromosome and thereby measured chromatin dynamics (Marshall *et al.* 1997) (see Table 3).

Taken together, the results obtained with these live cell methods demonstrate that chromatin can move by random diffusion as well as by ATP-dependent translocations whereby the rates and radius of gyration differ between species, and even between different cell types within a species, being influenced by parameters such as cell cycle, differentiation stage or position and size of the chromosomal marker (see Table 3) (also reviewed by Belmont 2003). New microscopy techniques that allow faster imaging combined with reduced phototoxicity, such as two-photon microscopy, should help to resolve the basis for these differences in the future (Levi *et al.* 2005).

Dynamics of chromatin binding proteins

In addition to all the features listed above, VFP fusion proteins can be used to directly measure chromatin protein diffusion and binding dynamics using techniques such as fluorescence recovery after photobleaching (FRAP) (reviewed in White & Stelzer 1999, Houtsmuller & Vermeulen 2001, Phair & Misteli 2001). In the FRAP method the VFP fluorescence signal is bleached by an intense laser beam followed by measurement of the fluorescence recovery into the bleached volume. One can distinguish several classes of molecules by this method, and quantitate their biophysical parameters using sophisticated analysis methods and simulations (Sprague *et al.* 2004, Ficz *et al.* 2005): (a) unbound,

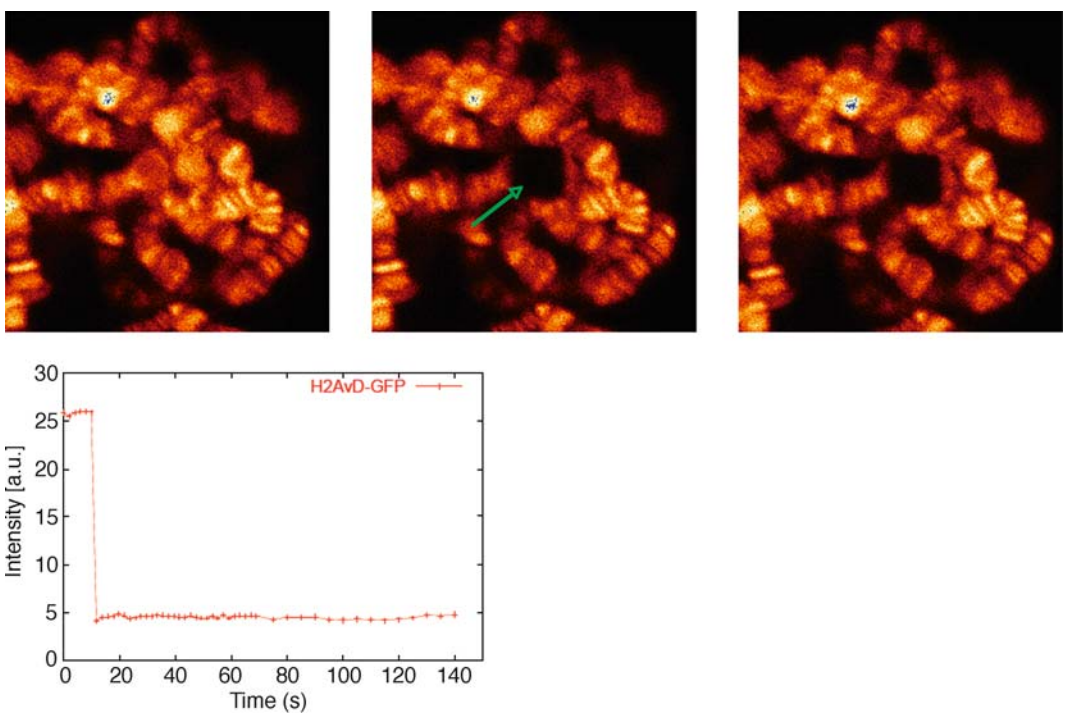
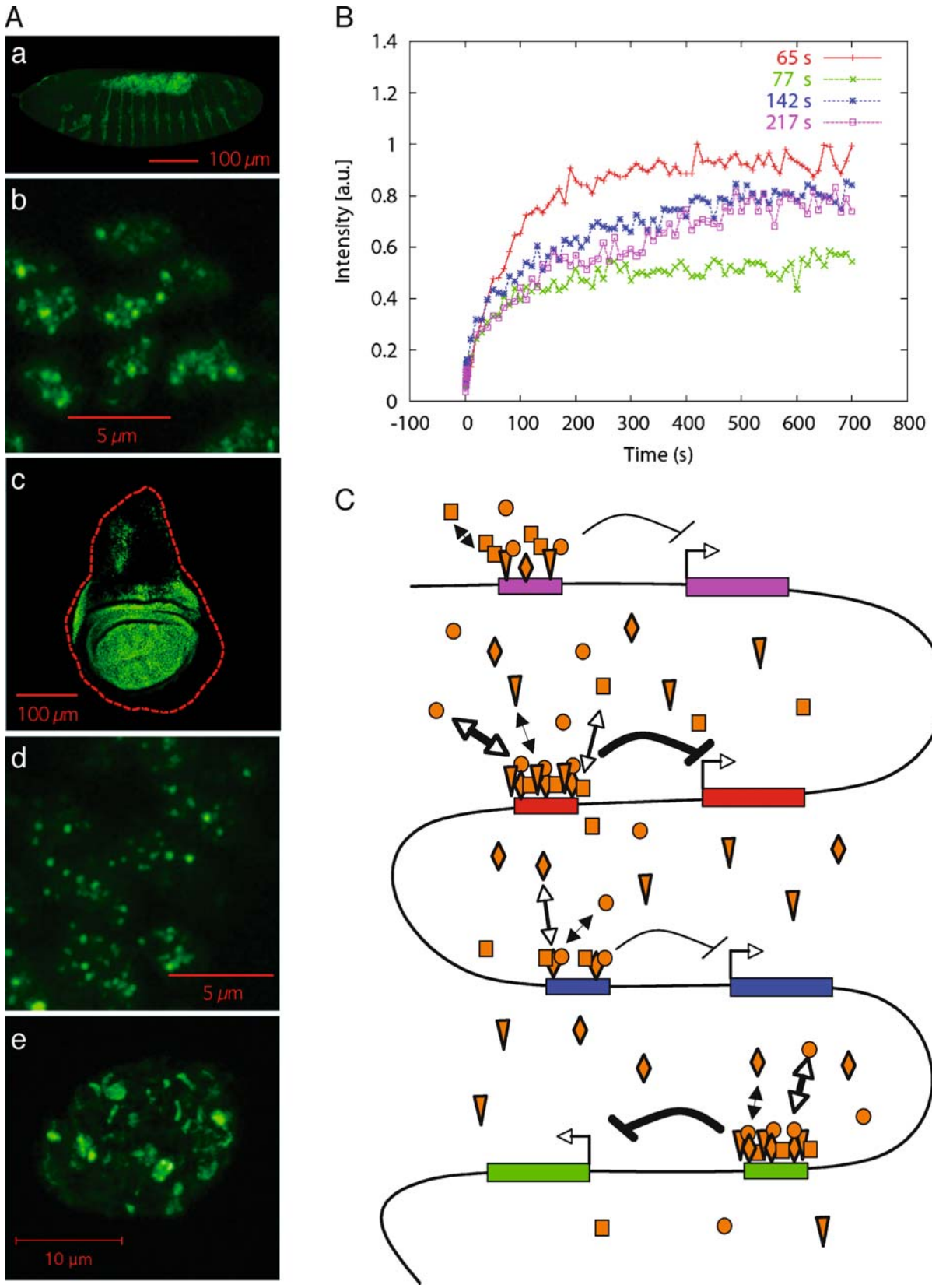


Figure 5. Core histones are stably integrated into chromatin and do not show exchange upon photobleaching. FRAP of H2AvD-GFP in a salivary gland nucleus. After bleaching there is no recovery of the bleached locus (green arrow). Panels left to right: prebleach image, first post-bleach image, last post-bleach image. Intensity trace within the box is plotted below for the whole period.


A
a
100 μm
b
5 μm
c
100 μm
d
5 μm
e
10 μm
B
65 s
77 s
142 s
217 s
Intensity [a.u.]
Time (s)
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freely diffusing molecules; (b) proteins involved in transient binding complexes on the chromatin; and (c) proteins that are tightly bound to chromatin and do not exchange with free protein during the time of the experiment. The core histones are the exceptional case of this latter category. They comprise the basis for DNA compaction and chromatin assembly and are tightly associated such that they can be used to visualize chromatin dynamics GFP- or paGFP fusions (see previous section, Figure 5, and Kimura 2005), whereas most other nuclear proteins interact only transiently with their DNA target sites. It has been found that even proteins involved in heterochromatin formation such as HP1 are exchangeable within 60 seconds (Cheutin *et al.* 2003). The polycomb group (PcG) proteins, Polycomb (Pc) and Polyhomeotic (Ph), were also shown to bind only transiently to their target sites (Ficz *et al.* 2005) although PcG proteins form large silencing complexes on chromatin (Shao *et al.* 1999) and are required throughout development to maintain silencing of their target genes (Beuchle *et al.* 2001). They are not trapped in the complex but rather are able to exchange completely within a few minutes. Additionally, the complexes have different residence times on individual bands in salivary gland nuclei, indicating that there are differences in the composition of the complexes on different genes (Ficz *et al.* 2005 and Figure 6).

It is to be expected that during the next decade new ways will emerge to combine many of the imaging techniques we have discussed above so as to obtain much more information about chromatin dynamics and the interplay between structure and function in the cell nucleus of the model developing organism *Drosophila*.

Materials and methods

DNA and other probes

Drosophila genomic DNA sequences were produced from P1 phage from the collection originally described by Hartl *et al.* (1994), but are denoted by their present numbers according to Flybase. The probes used for data described in this paper are P1 clones: *DS08009*, inside the *P115 3R* translocated segment but not in the *BX-C* complex; *DS04079*, lying on the non-translocated 3R segment distal to the P115 breakpoint; DS03126, part of the *Ultrabithorax* gene; *DS03648*, locus in the cytological band 89B11, without annotated genes, as well as a plasmid containing the histone gene cluster (kind gift of A. Udvardy). All DNA probes were labeled with digoxigenin by random priming using the High Prime Kit from Amersham Büchler (Braunschweig).

Monoclonal antibodies against msl-2 and lamin were the kind gifts of M. Kuroda and H. Saumweber, respectively.

Fly lines

For the transposition analysis *Tp(3;1) P115/w;Df/+* flies in which a segment containing the *BX-C* was translocated from the 3R to the X chromosome were used. As the transposition occurs in the X chromosome, males and females have to be distinguished from one another. A male-specific antibody (that recognizes the protein Msl-1, a kind gift from M. Kuroda) was used to distinguish male from female embryos. Only female embryos were analyzed.

The y^2 *w* scr^{t6} *uf; su(Hw)v/TM6B* and y^2*+;su(Hw)v, cas X/K/TM6B* strains were the kind gift of V. Corces and were crossed to obtain zygotic null mutants in the *su(Hw)* gene. These mutants show no Su(Hw) protein

Figure 6. Dynamic association of PcG proteins with PRE. **A**: Expression of a Ph-GFP fusion protein in a transgenic flyline. UAS-Ph-GFP expression was induced by Gal4 drivers in embryos (**a**,**b**), wing imaginal discs (**c**,**d**) or by a *Pc* promoter in salivary glands (**e**). **a**) Overview of an embryo showing Ph-GFP expression driven by the *en:Gal4* driver. Anterior to the left, dorsal to the top. (**b**) Ph-GFP is localized to many foci in embryonic nuclei. (**c**) Overview of a wing imaginal disc showing Ph-GFP expression driven by the Bx^{MS1096}*:Gal4* driver. Anterior to the right, dorsal to the top. (**d**) Ph-GFP is localized to many foci in larval diploid nuclei. (**e**) Ph-GFP expression under the *Pc*-promoter in a salivary gland nucleus. Ph-GFP is associated with bands on polytene chromosomes. **B**: FRAP curves for individual Ph-GFP binding bands in salivary gland nuclei. Individual bands show different recovery rates. **C**: Model for PcG protein function: PcG silencing complexes (orange) assemble at PRE. Individual PRE have different complex compositions resulting in different binding rates of the assembling proteins and different strengths of silencing.

in the imaginal disc and salivary glands in the third-instar larvae.

Imaging

All FISH imaging was carried out on either a Zeiss CLSM 310 or CLSM 510 with a Plan-Apochromat 63 × NA 1.4 oil immersion objective using simultaneous excitation of Cy5 anti-lamin at 633 nm and emission collected above 665 nm and excitation of Cy3 FISH signals at 514 or 543 nm respectively, emission bandpass 585 nm (HW 30). Image stacks were taken at 0.3 μm intervals (unless otherwise noted) with a pixel resolution of at least 0.1 μm. Pairing frequencies were calculated by visual inspection of the image stacks (results calculated by two different observers did not differ from the means) using Imaris (Bitplane, Zurich) image processing software. Interhomolog distances were calculated from Cartesian coordinates of the centers of FISH signal intensities, using scripts generated in Skil-image (Delft) or in 3D Viewer, a plug-in for Image J generated by R. Heintzmann. Optical sections of fixed whole mount embryos or larval discs were collected by multitrack imaging on the CLM 510 with the Plan-Apochromat 63XNA1.4 oil immersion of H2AvD-GFP (excitation 488 nm, emission 520 nm), Cy3 anti-lamin or anti-HP1 (excitation 543 nm, emission 585 nm), and Cy5 anti-lamin (excitation 633, emission above 665 nm).

In-vivo timed image stacks of 0.5 μm optical sections on live dechorionated embryos in buffered saline were acquired on a Zeiss CLSM 510 using excitation at 488 nm and emission at 520 nm with a 40 × 1.2 NA water immersion objective. Time-series were contrast stretched to compensate for photobleaching. No photodamage was detected since imaged embryos continued to develop normally. A segment of such a series is given as Supplementary Movie.

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