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Visualization of the genomic loci that are bound by specific multiprotein complexes by bimolecular fluorescence complementation (BiFC) analysis on *Drosophila* polytene chromosomes

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Abstract

We have developed a procedure that visualizes the genomic loci that are bound by complexes formed by a specific combination of chromatin-binding proteins. This procedure is based on imaging bimolecular fluorescence complementation (BiFC) complexes on *Drosophila* polytene chromosomes. BiFC complexes are formed by the facilitated association of two fluorescent protein fragments that are fused to proteins that interact with, or are in close proximity to, each other. The intensity of BiFC complex fluorescence at individual genomic loci is greatly enhanced by the parallel alignment of hundreds of chromatids within the polytene chromosomes. The loci that are bound by the complexes are mapped by comparing the locations of BiFC complex fluorescence with the stereotypical banding patterns of the chromosomes. We describe strategies for the design, expression and validation of fusion proteins for the analysis of BiFC complex binding on polytene chromosomes. We detail protocols for the preparation of polytene chromosome spreads that have been optimized for the purpose of BiFC complex visualization. Finally, we provide guidance for the interpretation of results from studies of BiFC complex binding on polytene chromosomes and for comparison of the genomic loci that are bound by BiFC complexes with those that are bound by subunits of the corresponding endogenous complexes. The visualization of BiFC complex binding on polytene chromosomes provides a unique method to visualize multiprotein complex binding at specific loci, throughout the genome, in individual cells.

1. Introduction

Protein interactions on chromatin are thought to regulate transcription in response to extracellular and intracellular signals. The mechanisms whereby such protein interactions produce the unique expression pattern of each individual gene remain incompletely characterized. In particular, the roles of protein interactions in determination of the genes that are bound by specific transcription factors are not known for the majority of proteins. Studies of genomewide binding by individual transcription factors have shown that many transcription factors can bind to the same regions (ENCODE Consortium, 2012). In most cases, it is not known if these proteins bind to the regions as complexes in the same cell or if

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they bind to the regions independently or in different cells. These questions are particularly difficult to answer in the case of tissues that contain many different cell types. Methods that enable the direct visualization of protein complexes that are bound to chromatin at specific genomic loci will provide valuable insight into the roles of protein interactions in the determination of the chromatin binding specificities of transcription regulatory proteins in individual cells.

Many methods have been developed to detect protein binding to specific genomic loci in cells (e.g. chromatin immunoprecipitation (ChIP), Dam-ID, DNase footprinting) (Galas and Schmitz, 1978; Gilmour and Lis, 1984; van Steensel and Henikoff, 2000). These methods have been adapted to determine the locations of protein binding throughout the genome (ChIP-seq, Dam-ID, DNase-Seq) (Boyle et al., 2008; Johnson et al., 2007; van Steensel et al., 2001). Most of these methods require a relatively large number of cells to determine the locations of protein binding (Table 1). These methods generally cannot distinguish if a protein binds to different genomic locations in the same cell or in different cells in a population. It is generally not possible to determine if two different proteins bind together as a complex or if they bind to the same location independently or in different cells. Sequential ChIP (re-ChIP) analysis can be used to determine if different proteins bind to the same chromatin segment (Hatzis and Talianidis, 2002). However, this does not distinguish if the proteins bind independently or as a complex.

Some methods enable direct visualization of protein binding to chromatin in individual cells by imaging proteins on chromatin (e.g. immunostaining or fluorescent fusion protein binding to polytene chromosomes; BiFC complexes with histones, immunofluorescence-FISH) (Lis, 2007; Masumoto et al., 1989; Schwartz et al., 2004; Silver and Elgin, 1976; Vincenz and Kerppola, 2008; Zhimulev et al., 2004). These methods can be used to detect chromatin binding by several proteins in the same cells (Schwartz et al., 2004; Shaffer et al., 2002). The limited spatial resolution of imaging prevents determination whether the proteins bind to the same loci as a complex or independently (Table 1).

Many important questions cannot be answered using the standard assays for the detection of protein binding to specific genomic loci. In particular, it is not possible to determine the chromatin loci that are bound by protein complexes that contain a specific combination of interaction partners. For this purpose, methods that enable simultaneous detection of protein interaction and of chromatin binding are required.

Bimolecular fluorescence complementation (BiFC) analysis enables visualization of protein interactions in living cells (Hu et al., 2002). In this assay, the interaction between two proteins is detected based on the fluorescence that is produced by the association of two fragments of a fluorescent protein that are fused to the two interaction partners (Fig. 1A). BiFC analysis has been adapted to enable the visualization of protein interactions in primary cells and in tissues (Bai and Kerppola, 2011; Diaz et al., 2005; Gohl et al., 2010; Hudry et al., 2011; Plaza et al., 2008). BiFC analysis has also been used to visualize epigenetic regulatory protein binding to large chromosomal regions on mitotic chromosomes in cultured cells (Vincenz and Kerppola, 2008). Because of the limited resolution of fluorescence microscopy, it has not been possible to map the specific genomic loci that are

bound by protein complexes by using BiFC analysis in diploid cells. To overcome this limitation, we adapted BiFC analysis to visualize protein complex binding at specific endogenous genomic loci on *Drosophila* polytene chromosomes (Fig. 3A) (Deng and Kerppola, 2014).

The visualization of BiFC complex binding on polytene chromosomes requires the selective expression of two fusion proteins in salivary glands at appropriate levels. Fusion proteins can be expressed in the salivary glands of transgenic *Drosophila* larvae by using the GAL4-UAS strategy (Brand and Perrimon, 1993). Using this method, BiFC complexes representing homodimers of the Scr homeodomain protein were detected in salivary gland cells, and were found to be enriched in a cluster in salivary gland cells that contain multiple tandem copies of a synthetic Scr binding site, consistent with chromatin binding (Papadopoulos et al., 2012). The endogenous genomic loci that are bound by fluorescent proteins can be mapped by comparing the locations of protein fluorescence with the banding patterns of polytene chromosomes (Deng and Kerppola, 2013). Likewise, the endogenous loci that are bound by BiFC complexes can be identified by comparing the locations of BiFC complex fluorescence with the polytene chromosome banding patterns (Deng and Kerppola, 2014). For this purpose, the conditions for spreading and imaging of polytene chromosomes must be optimized to preserve the fluorescence of BiFC complexes and to visualize the banding patterns of polytene chromosomes. Conditions for spreading and imaging of fluorescent fusion protein binding on polytene chromosomes have been developed (DiMario et al., 2006; Johansen et al., 2009). These conditions eliminate the acids that are used in conventional protocols that can quench the fluorescence of many fluorescent proteins. Using this strategy, we identified specific chromatin loci that are bound by BiFC complexes that are formed by dKeap1 (*Drosophila* Keap1) and CncC (*Drosophila* Nrf2 homologue) fusion proteins (Deng and Kerppola, 2014).

The use of BiFC analysis to visualize protein complex binding to polytene chromosomes enables direct detection of the genomic loci that are bound by protein complexes that contain a specific combination of interaction partners in individual cells. The approach can be used to compare the binding specificities of protein complexes and of individual proteins in parallel. This can identify mechanisms that affect the chromatin binding specificity of transcription regulatory protein complexes. The current approach has several limitations, including the requirement for polytene chromosomes that are found mainly in the salivary glands of *Drosophila*. The stable association of fluorescent protein fragments can also alter the dynamics of fusion protein interactions and could influence their binding to chromatin.

2 Design of fusion proteins for BiFC analysis

2.1

Fusion proteins that contain fragments of fluorescent proteins fused to the protein of interest are designed using principles that have been described previously (Hu et al., 2002; Hu and Kerppola, 2003; Kerppola, 2006). The fluorescent protein fragments can be fused to the N- or C-termini of the proteins of interest. The efficiency of BiFC complex formation often differs depending on the locations of the fluorescent protein fragments in the complex. It is therefore important to test many different combinations of fusion proteins to identify the

combination with the highest efficiency and specificity of BiFC complex formation. To reproduce the functions of the native proteins, it is best to avoid fusing the fragments near protein regions that have known functions. The functions of the fusion proteins should be tested using several independent assays that enable comparison of their functions with those of the native proteins.

2.2

It is generally best to design fusion proteins that encompass the complete proteins of interest. Fusion proteins can also be constructed that contain selected regions of the proteins of interest. The characteristics of such proteins may or may not correspond to a subset of the characteristics of the intact proteins. In all studies of protein interactions, it is important to include negative control proteins that contain substitutions that are predicted to disrupt the interaction. Such control proteins should be analyzed in parallel with, and under conditions identical to those that are used to analyze the intact proteins. Unrelated proteins are not useful controls since many of their characteristics are likely to differ from those of the proteins of interest.

2.3

Many different fluorescent protein fragments have been used to investigate protein interactions in cultured cells (Chu et al., 2009; Hu et al., 2002; Hu and Kerppola, 2003; Robida and Kerppola, 2009; Saka et al., 2007; Shyu et al., 2006). Fragments of different proteins have different characteristics that confer distinct advantages and disadvantages. We have used fragments of YFP that end at residues 155 and 173, and have obtained results that are consistent with facilitated complex formation by these fragments in *Drosophila* tissues when they are fused to interaction partners, but not when they are fused to non-interacting proteins (Deng and Kerppola, 2014). A variety of different fluorescent protein fragments have been used in studies with cultured mammalian cells, including fragments of the Venus fluorescent protein. BiFC complex formation by fusion proteins that contain fragments of Venus often results in a higher fluorescence intensity than BiFC complex formation by fusion proteins that contain YFP fragments. At least some combinations of fusion proteins that contain fragments of Venus produce spontaneous BiFC complexes in the absence of a specific interaction between the proteins that are fused to the Venus fragments (Fig. 1C) (Robida and Kerppola, 2009; Saka et al., 2007). The advantages and disadvantages of different fluorescent protein fragments in the investigation of protein interactions in *Drosophila* have not been established.

3 Construction and testing of plasmid vectors for BiFC analysis

3.1

The principal consideration in the choice of the strategy to be used for expression of the fusion proteins is the purpose of the experiments. This choice often involves a trade-off between a high level of expression to ensure that interaction partners form a level of BiFC complexes that can be detected and a lower level of expression that is more similar to, and therefore more likely to reflect the properties of, the endogenous proteins. The GAL4-UAS system enables fusion protein expression in specific tissues, including salivary glands. The

original version of this approach does not enable control of the level of protein expression, which is often much higher than that of the corresponding endogenous proteins. We have also used this strategy to express BiFC fusion proteins in the developing heart, muscle, and imaginal disc cells.

3.2

To express BiFC fusions using the GAL4-UAS system, plasmid vectors based on the pUAST plasmid need to be constructed (Brand and Perrimon, 1993). pUAST plasmids that encode the proteins of interest fused to fluorescent protein fragments are constructed by using standard molecular biology methods. Many different linker sequences have been used to connect different proteins to fluorescent protein fragments. The effects of different linker sequences on the efficiency and specificity of BiFC complex formation have not been tested directly. In this article, we describe results from experiments using dKeap1 and CncC proteins that are fused to the N-termini of YN173 and YC173 (Fig. 1B).

3.3

Because of the substantial effort that is required to generate *Drosophila* lines that express fusion proteins in specific tissues, it is best to test the efficiency and specificity of BiFC complex formation first in cultured cells. BiFC complex formation can be visualized in cultured *Drosophila* cell lines such as Schneider 2 (S2) cells (Fig. 1C). This can expedite the experiments by enabling use of the same plasmids for transient expression in cultured cells for the purpose of testing BiFC complex formation, and for the generation of transgenic *Drosophila* lines. The constructs can be transfected into S2 cells using Fugene 6 or other transfection reagents.

Important controls—Plasmids that encode the intact fusion proteins as well as fusion proteins in which residues that are essential for the interaction are substituted should be compared in parallel. Plasmids that encode fusion proteins that are known to produce BiFC complexes in the cells should be included as positive controls. The efficiency of plasmid introduction and expression in the cells should be determined by the expression of an intact fluorescent protein. The proportion of cells that express interaction partners that have detectable BiFC fluorescence is often less than 10% of the number of cells that express an intact fluorescent protein. The proportion of cells that express non-interacting proteins that have detectable fluorescence is expected to be at least an order of magnitude lower.

Troubleshooting 1. Result: Low frequency of fluorescent cells in transient expression assays—It can be difficult to transfect S2 cells with high efficiency. In general, low transfection efficiency is not a problem as long as the fluorescence intensity produced by a specific interaction is sufficiently high to be distinguished from that which is produced by non-interacting control fusion proteins. If the efficiency is not sufficient, several alternative strategies can be tried: 1) Construct additional plasmid vectors with different configurations of fluorescent protein fragments fusions; 2) Modify the method used for cell transfection; 3) Use a different cell line for transfection such as human embryonic kidney HEK293T cells.

Troubleshooting 2. Result: The intensity of BiFC fluorescence is very weak—

Many BiFC complexes form inefficiently and produce much lower fluorescence intensity than intact fluorescent proteins. The low fluorescence intensity is not a problem as long as it is sufficiently high to be distinguished from that which is produced by non-interacting control fusion proteins. If the fluorescence intensity is not sufficient, several alternative strategies can be tried: 1) Use a highly sensitive CCD camera and a long time of exposure; 2) Modify the method used for cell transfection; 3) Use a different cell line for transfection such as HEK293T cells; 4) Construct additional plasmid vectors encoding proteins in which the fluorescent protein fragments are fused to different positions of the putative interaction partners; 5) Use fluorescent protein fragments that produce BiFC complexes with higher efficiencies – note that some fragments, including fragments of Venus, can form BiFC complexes independently of a specific interaction (Fig. 1C) (Robida and Kerppola, 2009; Saka et al., 2007).

Troubleshooting 3. Result: Amino acid substitutions that are predicted to eliminate protein interactions do not reduce the BiFC complex fluorescence—

This could be due to either a low efficiency of BiFC complex formation by the intact fusion proteins or a high efficiency of BiFC complex formation by the mutant fusion proteins. To address the former issue, see troubleshooting 1 and 2 above. To address the latter issue, it is recommended that you use fluorescent protein fragments that have a low propensity to associate with each other in the absence of a specific interaction between proteins fused to the fragments (Robida and Kerppola, 2009). It is also possible that a high level of fusion protein expression favors complex formation by proteins that do not form complexes efficiently at lower concentrations. Finally, it is possible that the amino acid substitutions do not eliminate complex formation by the proteins of interest.

4. Production of *Drosophila* lines that express BiFC fusion proteins

4.1

Plasmids that express proteins of interest fused to fluorescent protein fragments as well as plasmids that express control proteins containing substitutions at residues that are predicted to be essential for the interaction are microinjected into *Drosophila* embryos. Transgenic lines are established and selected as described (Bachmann and Knust, 2008; Rubin and Spradling, 1982). Multiple independent lines should be produced in order to evaluate potential effects of the integration site on the levels of fusion protein expression and on BiFC complex formation. BiFC complex formation should be compared between lines that express the intact proteins and lines that express the mutant proteins at comparable levels.

4.2

The chromosomal locations of the transgenes are mapped as described (Bachmann and Knust, 2008). Some alleles can be deleterious, especially when homozygous. Such alleles should be maintained using appropriate balancer chromosomes (Robert, 1998). Complementary transgenes that are integrated on chromosomes 2 and 3 make the derivation of double transgenic lines most convenient. Lines that contain two transgenes on the same chromosome can be derived by homologous recombination (Robert, 1998). The transgenes

that express fusion proteins should be maintained as a stock separate from GAL4 transgenes to avoid potential selection for mutants that alter fusion protein expression.

4.3

The GAL4 driver transgene should be selected based on two criteria: (1) production of the lowest level of fusion proteins that are necessary for the detection of BiFC complex formation by interaction partners, and (2) specific expression in salivary glands or the tissue(s) of interest. Expression of the fusion proteins in many different tissues increases the risk that they have deleterious effects. Such expression could reduce the fertility of the flies, complicating the experiments.

4.4

To visualize protein complex binding to specific chromosomal loci, we expressed BiFC fusion proteins in the salivary glands of 3rd instar larvae. We used the Sgs3-GAL4 driver, which promotes UAS-controlled transcription selectively in salivary gland cells during the late 3rd instar stage (Cherbas et al., 2003). Other GAL4 drivers that promote transcription in salivary gland cells, including fkh-GAL4, 71B-GAL4 and 34B-GAL4, could potentially be used (Gerlitz et al., 2002; Maybeck and Roper, 2009). These drivers potentially promote higher levels of fusion protein expression in salivary glands or in other tissues. This can reduce the selectivity of BiFC complex formation and binding to specific genomic loci, and could have deleterious effects for the flies.

Troubleshooting 1. Result: The combination of two transgenes encoding BiFC fusion proteins has deleterious effects even in the absence of GAL4 driver expression—In the event that long-term maintenance of the two BiFC transgenes in the same line produces a deleterious phenotype, they can be maintained separately and lines that produce both fusion proteins can be produced by crossing one line first with the GAL4 driver line and subsequently with the other BiFC fusion protein line.

Troubleshooting 2. Result: The expression two BiFC fusion proteins has deleterious effects—In the event that short-term expression of the two BiFC transgenes under the control of a GAL4 driver has deleterious effects, alternate expression strategies can be used: 1) Test multiple transgenic lines – lines with lower levels of expression can have fewer deleterious effects; 2) Test GAL4 drivers that promote different levels of expression in different tissues; 3) Test inducible expression strategies, including GAL80ts, GeneSwitchGAL4, and GAL4-UAS combined with tet-on or tet-off systems (Han et al., 2000; Osterwalder et al., 2001; Stebbins et al., 2001; Zeidler et al., 2004) .

5. Comparison of the levels of BiFC fusion protein expression

5.1

The levels of fusion protein expression can affect the specificity of their interaction. It can also alter their subcellular localization, functions and chromatin binding. The GAL4-UAS system can produce levels of fusion proteins that exceed those of endogenous proteins by several orders of magnitude. To identify potential artefacts that are caused by fusion protein

overexpression, it is essential to compare the characteristics of the ectopically expressed fusion proteins with those of their endogenous counterparts. Usually different transgenic lines express different levels of the fusion proteins. Comparison of the results that are obtained using different transgenic lines with different levels of fusion protein expression can facilitate determination if differences in the levels of fusion protein expression affect the results.

5.2

To compare the levels of fusion protein expression with their endogenous counterparts, extracts of the tissues where they are expressed can be analyzed by immunoblotting. In many cases, fusion proteins are expressed at levels that exceed their endogenous counterparts by more than an order of magnitude (Fig. 2A). Ideally, the tissues that are analyzed should be matched with those where the GAL4 driver is known to be expressed.

5.2.1—To analyze the levels of fusion proteins in salivary glands, *Drosophila* larvae are prepared as described in part 5.

5.2.2—Carefully remove wandering 3rd instar larvae from the vial using tweezers. Briefly rinse the larva with water, and transfer the larva into PBS. Under a stereoscope, dissect salivary glands quickly as described in part 6.3.

5.2.3—Immediately transfer the salivary gland into 30 μ L IP buffer (with freshly added protease inhibitors) in a 0.2 mL glass tissue grinder (KONTES) on ice. Dissect additional larvae to harvest 3–6 pairs of salivary glands in 30 μ L IP buffer per protein whose expression is to be analyzed.

5.2.4—Apply pre-chilled pestle to grind tissue 10 times on ice. Transfer the sample into a 0.5 mL tube. Add 7 μ L of 5x SDS-loading buffer and mix well. Boil for 10 minutes. Clear the lysate by centrifuging for 1 minute at > 12000 g at room temperature. Load the supernatant onto an SDS-PAGE gel, or store at -80°C .

5.2.5—Analyze the cleared lysate using SDS-PAGE followed by immunoblotting, as described (Wang et al., 2001). The fusion proteins and their endogenous counterparts are detected using antibodies directed against the endogenous proteins. Anti-tubulin antibody should be used to evaluate the amounts of protein loaded in different lanes.

Troubleshooting. Result: Weak or undetectable signals on immunoblotting: The levels of many endogenous proteins are difficult to detect in salivary glands. The following approaches can be used to improve their detection: 1) Ensure optimal immunoblotting protocol and time of exposure. Use proteins from other sources (recombinant, cultured cells) to optimize the assay; 2) Use larger numbers (10–20) of salivary glands for extraction; 3) Ensure efficient extraction of the proteins from the tissue by evaluating the extraction of other endogenous protein such as tubulin or lamin Dm0.

5.3

The subcellular localization and the chromatin binding specificities of the fusion proteins should be compared with their endogenous counterparts (Fig. 2B). This should be done for each of the fusion proteins expressed separately and in combination. Differences in the subcellular localization or chromatin binding by the fusion proteins compared to their endogenous counterparts should be considered carefully when interpreting results of BiFC analysis. Differences in the subcellular localization or chromatin binding of fusion proteins when they are expressed in combination versus separately could reflect effects of their interactions on their localization or chromatin binding.

5.3.1—To determine the subcellular localization of fusion proteins in salivary gland cells, *Drosophila* larvae are prepared and their salivary glands are excised from the larvae as described in parts 5 and 6.3. In general, it is not necessary to completely remove mouth hooks and other attached tissues (brain, imaginal discs). The salivary glands are fixed and immunostained using antibodies directed against the fluorescent protein fragments and the endogenous proteins as described (Phalle Bde, 2004).

5.3.2—The immunostained salivary glands are imaged by fluorescence microscopy. The distributions of the fusion proteins in salivary glands from the transgenic larvae are compared with the distributions of the endogenous proteins in salivary glands from wild type larvae.

Troubleshooting. Result: Weak or undetectable immunofluorescence signals: The levels of many endogenous proteins are difficult to detect in tissues. Use a sensitive CCD camera and a long exposure time. Optimize the immunostaining protocol by varying the concentrations and incubation time of primary and secondary antibodies. Analyze proteins that are known to be present at high levels in salivary glands, or other tissues known to contain high level of target proteins in parallel as controls.

6. Preparation of *Drosophila* larvae for salivary gland isolation

6.1

To produce larvae that express BiFC fusions in the salivary glands, cross the Sgs3-GAL4 driver line with a double transgenic BiFC fusion protein line (e.g. Sgs3-GAL4/Sgs3-GAL4 × UAS-dKeap1-YC/UAS-dKeap1-YC; UAS-CncC-YN/UAS-CncC-YN). Either male or female flies carrying the BiFC transgenes can be crossed with female or male flies carrying the GAL4 driver. Ideally, equal numbers of male and female larvae should be used for optimal mating efficiency. In cases where limited numbers of flies of one sex are available a ratio of up to 1:5 males:females can be used. We prefer crossing male flies carrying the BiFC transgenes to female flies carrying the GAL4 driver since: 1) The GAL4 transgenic females generally have high fertility, which can increase the number of progeny; 2) These females can be readily amplified to perform multiple crosses with males that carry different combinations of BiFC transgenes to compare BiFC complex formation by different combinations of fusion proteins.

6.2

To enhance replication of the polytene chromosomes to high copy number and to improve polytene chromosome spreading, culture the F1 progeny larvae at 21°C under conditions of minimal crowding (place 4 females and 4 males in a vial and change the vial every day). Larvae normally climb up the sides of the vial within 6–7 days after mating. Select the fattest 3rd instar wandering larvae to emerge early, and after the mating. The larvae should be harvested immediately before pupation. Pupation is preceded by reduced mobility of the larvae on the wall of the vial. Larvae can also be raised at 18°C, which can increase the thickness of the polytene chromosomes (Johansen et al., 2009). An additional benefit of the lower temperature is that it reduces transcription of the transgenes, which can reduce the levels of fusion protein expression.

Troubleshooting 1. Result: Very few or no progeny are produced—If no progeny are produced in multiple matings, it is likely that either one of the lines is not fertile. Test each line with wild type flies in parallel to verify the conditions and to identify the infertile line. If the double transgenic BiFC fusion line is infertile, mate the two transgenic lines encoding different BiFC fusions sequentially to the GAL4 line. Also, try to switch the sexes of the flies that carry the various transgenes.

Troubleshooting 2. Result: 3rd instar larvae that express BiFC fusions have small salivary glands—Overexpression of some proteins can inhibit the growth or cause the death of salivary gland cells prior to full amplification of the polytene chromosomes. To overcome this problem, test lines with lower levels transgene expression. If necessary, protein expression can be regulated using GAL80ts, GeneSwitchGAL4, or tet-on/off systems (Han et al., 2000; Osterwalder et al., 2001; Stebbins et al., 2001; Zeidler et al., 2004).

7. Preparation of polytene chromosome spreads

7.1

Isolation and fixation of salivary glands

7.1.1—In the conventional squash protocol, acetic and lactic acids are added to facilitate chromosome spreading. This increases the resolution of the banding patterns. These acids quench the intrinsic fluorescence of YFP and BiFC complexes. To avoid quenching of the BiFC fluorescence, polytene chromosome spreads should be prepared using an acid-free squash protocol (DiMario et al., 2006; Johansen et al., 2009). In this procedure, salivary glands are fixed in dilute formaldehyde and squashed after incubation in 50% glycerol. The high viscosity of the squash medium helps preserve the chromosome morphology during spreading (Johansen et al., 2009).

7.1.2—Carefully remove a wandering larva from the vial using tweezers. Briefly rinse the larva with water in a dish. Transfer the larva into 2 ml of freshly made Brower's Fixation Buffer in a Petri dish at room temperature.

7.1.3—To isolate and fix salivary glands, under a dissecting microscope, dissect the larva as follows (Johansen et al., 2009). Using two pairs of tweezers, grasp the mouth hooks and hold the body at a position about 2/3 of the way down. Pull on the mouth hooks to open the body and expose the salivary glands. Remove the brain, imaginal discs, and fat tissue surrounding the salivary glands. The dissection should be completed during fixation, which generally takes 2–3 minutes. The time of fixation may need to be optimized for different proteins. Transfer the salivary gland into 2 ml PBST (see section 12) and incubate 2–3 minutes. Then transfer into 2 ml of 50% glycerol and incubate 5 minutes at room temperature.

7.2

Salivary gland squash to spread polytene chromosomes

7.2.1—Use Kimwipes to clean siliconized coverslips and poly-lysine coated microscope slides. Transfer the salivary gland into 15 μ l of 50% glycerol on the coverslip. Use tweezers to fragment the salivary glands into 10–20 pieces. Carefully lower a slide onto the coverslip, and flip it after the coverslip attaches to the slide. Using the eraser of a pencil, gently move the coverslip back and forth about 2 mm 3–5 times to separate and lyse the cells and to spread the chromosomes. Apply a light pressure to the eraser that is just enough to move the coverslip. Clouding of the sample is an indication that the cells have separated and lysed. Tap the coverslip with the eraser of a pencil or the back of forceps to encourage chromosome spreading. One technique is to drop a pencil from a height of about 10 cm on the eraser end onto the coverslip. Repeat 10–20 times. Hold one corner of the coverslip to prevent large horizontal movements as this can damage the polytene chromosomes.

7.2.2—Examine the preparation immediately using a phase contrast microscope with a 20x objective. If many of the chromosomes appear to be well-spread, proceed to the next step. If not, tap the coverslip again and continue tapping until an appropriate extent of spreading is obtained. Under the acid-free conditions, spreading of the polytene chromosomes is hindered by cross-linking, making the chromosomes more difficult to spread. Therefore, the acid-free protocol requires more and harder tapping compared with conventional squash protocols. This often results in broken chromosome arms. The broken chromosome arms can often be used to map individual genomic loci as long as they have clear banding patterns (Fig. 3B). A larger number of squash attempts is needed to obtain intact polytene chromosome preparations using the acid-free squash protocol than when the conventional protocol is used.

7.2.3—Place the slide between two layers of tissue paper. Flatten the chromosomes by placing your thumb on the coverslip and press firmly. Avoid horizontal movement of the coverslip since this can shear the chromosomes. Fill a thermal flask with liquid nitrogen. Hold the slide with a long forceps, put the slide in the liquid nitrogen until the boiling stops. Remove the slide and immediately (before the disappearance of frost) use a new razor blade to flip off the coverslip from the slide. Place the slide in a Coplin jar with cold PBS. If more slides are prepared, store the slides in PBS at 4°C for up to 2 hours.

Troubleshooting 1. Result: Inadequate spreading of polytene chromosomes: The polytene chromosomes can be hard to spread. Make sure that the salivary glands are well developed with lengths corresponding to $1/3 - 1/2$ of the body length. Use freshly prepared fix solution within 10 minutes after preparation. Optimize the fixation time: A shorter fixation time (2 minutes or less) can improve spreading as long as it is sufficient to cross-link the BiFC complexes to the chromatin.

Troubleshooting 2. Result: A small number of polytene chromosome spreads is found on the slides: Check the coverslip that was removed from the frozen slide. If polytene chromosomes are attached to the coverslip (indicated by a non-reflective surface), prepare new siliconized coverslips and poly-L-lysine coated slides.

8. Staining and imaging of polytene chromosomes

8.1

Prior to staining the sample, briefly tap the edge of the slide to remove excess PBS. Place the slide tissue side up in a humidity chamber. Apply 100 μ l of Hoechst solution on the tissue. Cover the tissue with a 20×35 mm piece of parafilm to fully cover the sample. Cover the chamber and incubate 10 minutes in the dark at room temperature.

8.2

Remove the Parafilm and tap the slide to remove the Hoechst solution. Return the slide into the Coplin jar and incubate 2×5 minutes in PBS in the dark. Drain the slide by touching the edge to a tissue paper and dry the back. Remove as much liquid as possible without touching the tissue. Air dry the slide in the dark for 1–2 minutes.

8.3

Place 1–2 drops (10–20 μ l) of mounting solution on the tissue. Touch one edge of a 22×40 mm coverslip to the solution and lay down slowly to avoid bubbles. Leave the slide in dark for 5 minutes to allow the mounting solution to permeate the sample. Wick off excess mounting solution using tissue paper. Seal the edges of the coverslip using nail polish.

8.4

The polytene chromosomes with BiFC complexes can be co-stained using antibodies directed against endogenous or ectopic proteins (Silver and Elgin, 1976). Secondary antibodies conjugated to fluorophores whose spectra do not overlap with that of the BiFC complexes should be used.

8.5

Images of the polytene chromosomes are acquired using a fluorescence microscope. The locations of BiFC complexes and the banding pattern of Hoechst staining are imaged using different excitation and emission wavelengths. BiFC complexes formed by YFP fragments can be imaged using 504 nm excitation and 542 nm emission wavelengths. Hoechst signal can be imaged using 358 nm excitation and 461 nm emission wavelengths. The best results are obtained by imaging the samples immediately after preparation. If the samples must be

stored, store them at -20°C in the dark. To distinguish BiFC complex fluorescence from cross-talk with other fluorophores and other potential sources of fluorescence, samples that do not contain both fusion proteins should be processed and analyzed in parallel.

8.6

Polytene chromosomes that are visualized using the acid-free protocol generally have less sharp banding patterns than those that are produced by conventional squash protocols (Fig. 3C). It is often necessary to perform a larger number of squashes to produce satisfactory data. Persistent effort to optimize the squash procedure is generally rewarded with spreads that enable mapping of BiFC complex binding throughout the genome.

Troubleshooting. Result: Weak or no BiFC fluorescence is detected—In general, the fluorescence intensity of BiFC complexes is much weaker than those of intact fluorescent proteins that are expressed at similar levels. This is likely to be due to the fact that only a small portion of fusion proteins form BiFC complexes (Hu et al., 2002). To avoid bleaching weak BiFC complex fluorescence, samples should be initially viewed by excitation of Hoechst fluorescence to locate chromosome spreads and to adjust focus. A sensitive CCD camera and long exposure times are often necessary. The use of fluorescent protein fragments with brighter BiFC fluorescence can help. However, these can result in complex formation by proteins that do not normally interact with each other (Fig. 1C) (Robida and Kerppola, 2009). Likewise, higher levels of protein expression can increase the fluorescence signal. However, higher levels of fusion protein expression can result in complex formation by proteins that do not normally interact with each other as well as potential deleterious effects.

9. Interpretation of BiFC complex binding on polytene chromosomes

9.1

The genomic loci that are bound by BiFC complexes can be determined by comparing the locations of BiFC complex fluorescence with the banding pattern that is produced by Hoechst staining (Fig. 3B,C) (Deng and Kerppola, 2014). The reproducible pattern of Hoechst staining allows determination of the chromatin bands and interbands that are occupied by BiFC complexes in individual cells. This approach enables mapping of the genomic locations that are bound by BiFC complexes at a resolution of 100–700 kb, which typically encompasses 10–40 genes depending on the genomic region.

9.2

To identify the specific genes that are bound and potentially regulated by the protein of interest, additional approaches can be used. Chromatin binding can be analyzed by chromatin immunoprecipitation if the complex binds the same region in a large proportion of the cells and if the location of the region is known or can be predicted with a resolution of 1 kb. In addition, the effects of depletion or overexpression of the proteins of interest on transcription of the genes within the chromatin region implicated by mapping of the location of BiFC complex binding can identify genes whose transcription is affected by the proteins of interest.

Troubleshooting. Result: Poorly resolved bands prevent mapping of loci bound by BiFC complexes—The polytene chromosome arms obtained by the acid-free squash protocol are generally more diffuse and less extended compared with the sharp and well-separated bands produced by conventional squash protocols (Fig. 3C) (Johansen et al., 2009). Optimization of the conditions of larval culture to produce maximally amplified polytene chromosomes and of the squash procedure will improve the quality of data that can be obtained. Larvae that are raised at lower temperatures (16–18°C) can produce more highly amplified polytene chromosomes. A shorter fixation time can improve polytene chromosome spreading and thus increase the quality of bands. The reproducibility of the squash procedure can be improved by the use of mechanical aids (Johansen et al., 2009; Novikov et al., 2007).

10. Strategies to limit the artefacts caused by the differences between fusion proteins and endogenous counterparts

10.1

Ectopically expressed fusion proteins can have subcellular distributions, chromatin binding specificities, or protein-interaction specificities that differ from those of their endogenous counterparts. These differences complicate the interpretation of potential effects of their interactions. There are several potential causes for differences between the characteristics of fusion proteins and their endogenous counterparts.

10.2

Expression of fusion proteins at levels that are much higher than the levels of their endogenous counterparts can alter their functions. The GAL4-UAS system provides little control over the level of fusion protein expression. This strategy often results in much higher levels of transgene expression than the levels of endogenous gene expression (Fig. 2A). The levels of transgene expression can be reduced by: 1) Screening for lines with a lower level of transgene expression. The eye color that is produced by the mini-white transgene can be used to pre-screen the lines since it reflects the chromatin environment of the integration site; 2) Raising the larvae at a lower temperature (16°C) to reduce the activity of GAL4 (Duffy, 2002); 3) The use of GAL4 drivers that are known to promote lower levels of transgene expression; 4) The use of inducible expression strategies, such as GAL80ts, GeneSwitchGAL4, or tet-on/off systems; 5) The use of native promoters to express the fusion proteins (Ding et al., 2009; Rugjee et al., 2013).

10.3

The fusion of fluorescent protein fragments to the proteins of interest can alter their characteristics. To reduce the risk that the fluorescent protein fragments affect the functions of the complex, the fragments can be fused to locations that are far from known functional domains. The use of longer or different linker sequences can also reduce the effects of the fluorescent protein fragments on complex functions.

11. Effects of BiFC complex formation on the specificity of chromatin binding

11.1

The association of the fluorescent protein fragments generally stabilizes the interaction of the fusion proteins to produce a complex that is more stable than complexes formed by the endogenous proteins. This stabilization could affect the specificity or the avidity of protein complex binding to chromatin.

11.2

To distinguish between the potential effects of BiFC complex formation and of fusion protein co-expression on the specificity of chromatin binding, the loci that are bound by BiFC complexes can be compared with those that are bound by the proteins of interest fused to intact fluorescent proteins when they are co-expressed (Deng and Kerppola, 2014). If the proteins that are fused to intact fluorescent proteins bind together at the same loci that are bound by the BiFC complexes, it is likely that BiFC complex binding reflects that binding specificity of the protein complex and is not affected by the association of the fluorescent protein fragments (Deng and Kerppola, 2014).

Troubleshooting. Result: BiFC complexes bind to loci that are not bound by endogenous proteins—Many factors can alter the specificity of chromatin binding by BiFC complexes. Artefactual effects include potential effects of the protein fusions on chromatin binding by the proteins of interest, and potential effects of ectopic overexpression of the fusion proteins on their expression levels. These can be evaluated as described above. The possibility that fluorescent protein fragment association affects chromatin binding directly can be evaluated by parallel analysis of chromatin binding specificity using other experimental approaches (Cai et al., 2014; Deng and Kerppola, 2013, 2014; Rugjee et al., 2013; Wang et al., 2001).

12. Materials and Solutions

12.1

Equipment and Materials

Dissecting microscope with a 5–10× objective

Illuminator

35 × 10 mm Petri dish

Drummond dissection tweezers (2) (72695-D)

0.2 ml Glass tissue grinder (Kimble 885470-0000)

Frosted microscope slides (Fisher 12–544-7) coated with poly-L-lysine (Sigma P8920).

Incubate slides in the poly-lysine solution for 5 minutes and dry the slides at room

temperature. Coated slides can be stored at room temperature for one year. Poly-lysine-coating increases the attachment of chromosome preparations to the glass surface.

Siliconized 22 × 22 mm 1.5 cover slips (Fisher No. 12–520B) coated with Sigmacote (Sigma SL2). To coat, immerse clean cover slips in Sigmacote in a Petri dish, then air dry in a fume hood. The siliconized cover slips can be stored at room temperature for at least one year. Clean with water and dry with Kimwipe before use. Siliconization prevents the attachment of chromosome preparations to the cover slip.

22 × 40 mm 1.5 cover slips (Fisher 12–544-B)

Tissue paper and Kimwipes

Pencil with eraser (or similar tools that can be used to tap the slides)

Phase contrast microscope with ~ 20x objective

Thermoflask with 300–500 mL of liquid nitrogen

Long forceps

Razor blades

Coplin Jar

Humidity chamber (Rubber-Maid or similar sealable tray. Cover bottom with moistened tissue papers. Use Pasteur pipets to support slides.)

Parafilm (cut into 22 × 35 mm pieces.)

Nail polish (to seal cover slips.)

12.2

Solutions

IP buffer (20 mM Tris-HCl (pH8.0), 150 mM NaCl, 10mM EDTA, 1mM EGTA, 0.2% TritonX-100, 0.2% NP-40, 2mM Na₃VO₄, 1mM PMSF, 1.5 µg/ml Aprotinin)

5x SDS loading buffer (0.25 M Tris-HCl (pH6.8), 0.5 M DTT, 50% Glycerol, 10% SDS)

37% formaldehyde (Fisher BP531)

Brower's fixation buffer (0.15 M PIPES, 3 mM MgSO₄, 1.5 mM EGTA, 1.5% NP40, adjust pH to 6.9, add formaldehyde to a final concentration of 2% just before use)

PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄)

PBST (PBS with 0.1% Triton X-100)

Hoechst 33258 (Molecular Probes) solution (0.2 µg/mL in PBS, store in the dark at 4°C)

Mounting solution (80% glycerol, 10 mM Tris pH 9.0, 5% n-propyl gallate) (avoid using anti-fade mounting solutions as they can quench fluorescent proteins.)

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Fig. 1.

(A) Diagram of BiFC complex formation by proteins fused to fluorescent protein fragments (YN and YC) (modified from Deng and Kerppola 2014); (B) Plasmids encoding dKeap1 and CncC fused to fluorescent protein fragments (YN and YC) were constructed using the pUAST vector (Brand and Perrimon, 1993). The pUAST vector contains five tandem GAL4 binding sites (UAS) that mediate transgene activation by GAL4 and sequences that facilitate transcription and P-element sequences that facilitate transgene insertion into the *Drosophila* genome. (C) BiFC analysis of the specificity of association of FKBP and FRB fusions to fragments of the Venus (VN173) and YFP (YC173) fluorescent proteins in S2 cells. The cells were cultured in the absence (–) and in the presence (+) of rapamycin, which induces FKBP-FRB interaction (Rivera et al., 1996). The fluorescence that is observed in the absence of rapamycin indicates that the fluorescent protein fragments associate spontaneously. Scale bars: 2 μ m.

Fig. 2. Comparison of the levels of protein expression and subcellular localization of dKeap1 and CncC fusion proteins that are expressed at different levels with their endogenous counterparts. (A) The salivary glands of larvae that express the fusion proteins indicated above each lane were excised and the levels of protein expression were analyzed by immunoblotting using the antibodies indicated to the right of each image. The endogenous dKeap1 and CncC proteins were not detected in this exposures. The bands representing dKeap1 (blue) and CncC (red) fusion proteins are labeled by arrowheads. Image modified from Deng and Kerppola 2014. (B) The salivary glands of wild type larvae (left column) and of larvae that expressed the fusion proteins indicated at the top of the images (right column) were analyzed by immunofluorescence (yellow) using the antibodies indicated at the bottom of the images. The nuclei were labeled by Hoechst staining (blue). Scale bars: 10 μm.

Fig. 3.

Visualization of BiFC complexes on polytene chromosomes. (A) Diagram of BiFC complex formation on polytene chromosomes. The intensity of BiFC complex fluorescence at individual genomic loci is enhanced by the parallel alignment of hundreds of chromatids within the polytene chromosomes. (B) Examples of BiFC complex fluorescence (green) on polytene chromosomes stained using Hoechst (blue). The chromosomes were spread under acid-free conditions. The banding patterns of the polytene chromosomes stained using Hoechst are shown in the images to the right. Note the differences in chromosome spreading and extension that are necessary to map the localization of the BiFC complexes to individual genomic loci. Upper panel: an intact spread allowing mapping of a number of loci, including the two copies of the 55F locus, corresponding to the two homologues that were presumably separated during the squash procedure; Middle panel: a partial spread with broken arms, which are well extended and suitable for mapping a subset of the loci; Lower panel: a poorly extended spread on which the loci that are bound by BiFC complex cannot be easily identified. Scale bars: 10 μ m. (C) Comparison of the localization of BiFC complexes and of the individual BiFC fusion proteins using acid-free (upper images) and conventional (middle and bottom images) squash protocols. Polytene chromosomes that prepared using the acid-free protocol have less sharp banding patterns than those that are prepared using conventional squash protocols. Both dKeap1-CncC BiFC complexes and each of the fusion proteins

bound the 55F locus. Both dKeap1 and CncC fusions bound many other loci, whereas the dKeap1-CncC BiFC complex bound the 55F locus with higher specificity. Scale bars: 5 μ m.

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Table 1

Comparison of the characteristics of different methods for genomewide identification of the locations of protein and protein complex binding

method	BiFC on polytene chromosome	BiFC with histones	IF on polytene chromosome	ChIP-seq	Dam-ID	DNase-Seq
characteristic						
target	specific protein complex	specific fusion protein	specific endogenous protein(s)	single endogenous protein	single fusion protein	any chromatin binding protein
resolution	hundreds kb	chromosome regions	hundreds kb	~100–1000 bp	~200–500 bp	~10–100 bp
sample size	single cells	single cells	single cells	cell population	cell population	cell population
potential caveats	exogenous BiFC fusion protein expression	exogenous BiFC fusion protein expression	antibody specificity	antibody specificity, signal amplification	exogenous Dam fusion protein expression, signal amplification	DNase I specificity, signal amplification
applicability	Drosophila salivary glands	cells with exogenous protein expression	Drosophila salivary glands	sequenced genomes	sequenced genomes	sequenced genomes