Cross-over analysis in fluorescent pollen tetrads
Methods for detecting superoxide produced in cells
Southwestern blotting to identify DNA-binding proteins
Live imaging and FRAP analysis of Drosophila synapses
Cyclodextrin synthesis for chiral separation
Fluorescent *Arabidopsis* tetrads: a visual assay for quickly developing large crossover and crossover interference data sets

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In most organisms, one crossover (CO) event inhibits the chances of another nearby event. The term used to describe this phenomenon is ‘CO interference’. Here, we describe a protocol for quickly generating large data sets that are amenable to CO interference analysis in the flowering plant, *Arabidopsis thaliana*. We employ a visual assay that utilizes transgenic marker constructs encoding pollen-expressed fluorescent proteins of three colors in the *quartet* mutant background. In this genetic background, male meiotic products—the pollen grains—remain physically attached thereby facilitating tetrad analysis. We have developed a library of mapped marker insertions that, when crossed together, create adjacent intervals that can be rapidly and simultaneously screened for COs. This assay system is capable of detecting and differentiating single COs as well as two-, three- and four-strand double COs. We also describe how to analyze the data that are produced by this method. To generate and score a double interval in a wild-type and mutant background using this protocol will take 22–27 weeks.

INTRODUCTION

Meiosis is a specialized type of cell division in which a prior round of DNA replication is followed by two successive rounds of chromosome segregation. The resulting cells have half of the original chromosomal complement. During fertilization, these cells fuse to reconstitute the original complement thereby completing the sexual cycle. In most eukaryotes, a feature of the first meiotic division (MI) is crossing over, in which homologous chromosomes interact physically, exchanging material between the paternal and maternal copies. Crossing over provides a connection between homologs that is required in most organisms for the accurate segregation of chromosomes during MI (ref. 2). Chromosomes that fail to have at least one crossover (CO) often segregate aberrantly, resulting in aneuploidy. In addition, crossing over generates genetic diversity by creating new combinations of paternal and maternal alleles.

Cells undergoing meiosis regulate recombination at multiple levels. To ensure that each chromosome pair is physically connected, COs are distributed among chromosomes nonrandomly such that each chromosome pair typically undergoes at least one CO even if the total number of COs per chromosome is less. The distribution of COs along chromosomes is also tightly regulated. In most organisms, COs are distributed such that one CO event inhibits the chances of another nearby event. The term used to describe this phenomenon, first observed by *Drosophila melanogaster* researchers at the beginning of the twentieth century, is CO interference.

One of the challenges of studying CO interference is that it is a probabilistic phenomenon of populations. CO interference does not result in a complete lack of closely spaced COs, but instead reduces the proportion of these events in a population compared with what one would expect if the events were randomly positioned. For this reason, making statistically significant claims often requires large data sets. Patterns of CO interference vary widely from organism to organism with regards to strength versus distance relationships and in some organisms there appear to be both interfering and noninterfering COs. Even within organisms, parameters such as sex and chromosomal location have been shown to have a profound effect on CO interference.

Using *Arabidopsis thaliana* as a model, we present here a fluorescent pollen tetrad method for quick and relatively easy production of data sets that can be used to analyze CO interference. Using this method, one can assay almost any chromosomal region, customize interval size and examine the effects of mutants and experimental treatments on CO interference. This method has been used to assay wild-type levels of CO interference on a region of chromosome 5 and also to determine a small but detectable difference in CO interference between wild-type plants and *Atmus* mutant plants.

Measuring multiple COs simultaneously

In addition to the fluorescent tetrad method described here, several other methods exist for measuring multiple COs simultaneously. These methods can be classified into two general categories: genetic, in which the researcher monitors the segregation of markers that define intervals, and cytologic, in which the researcher uses microscopy techniques to visualize structures that mark COs.

Genetic methods. These generally involve a crossing scheme in which an individual, heterozygous for multiple markers, gives rise to a set of progeny that possess chromosomes reflecting the recombination history of the parent. In some organisms, researchers can also monitor recombination directly in the products of meiosis by analyzing markers in such cell types as fungal spores or sperm of mammals. Genetic systems in which the four meiotic products are fused in a tetrad (or octad in some cases) are especially powerful because the researcher can account for all of the parental genetic material in each unit of data rather than analyzing a pool of random meiotic products. This allows,
among other things, the identification of nonparental diatype (NPD) tetrads, in which a four-strand double CO (DCO) has occurred in a single interval. Genetic methods allow for the unambiguous characterization of COs in multiple intervals, but the generation of large data sets can be time- and labor-intensive. The analysis of genetic markers can involve PCR genotyping, DNA blot hybridization, or a combination, which has to be done for every marker in each individual.

**Cytological methods.** These entail looking at structures on chromosomes that are known to be involved in the CO process. The most common method is immunostaining the meiotic prophase I chromosome spreads using antibodies for proteins that are known to be present in the complexes responsible for the formation of COs, such as Mlh1p. Because these proteins are part of the complex present at CO sites, the antibodies that bind to these proteins form foci, which can be counted. Often, this is done in conjunction with a differently labeled antibody that recognizes the synaptonemal complex so that the chromosomes can be visualized and distinguished. This method allows the researcher to monitor all chromosomes at once in each meiotic spread, but only offers a snapshot in time, which may not be indicative of all of the COs that will form. In addition, closely spaced double COs can be potentially difficult to resolve. Other cytological methods for interference analysis include the monitoring of late recombination nodules (LNs) and chiasmata. LNs are electron-dense structures that form during the late meiotic prophase I and are generally thought to represent CO complexes that will eventually become chiasmata. The correlation between LNs and COs is thought to represent CO complexes that will eventually become chiasmata. The correlation between LNs and COs is based on the observation that LN frequency and positional data closely matches that of genetic CO data. Chiasmata are mature COs that have created physical linkages between homologous chromosomes before MI division, which can be visualized in diplotene. These entail looking at structures on chromosomes that are known to be involved in the CO process.

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**Fluorescent tetrad analysis in A. thaliana**

Our lab has developed a unique pollen-based visual assay for meiotic recombination in A. thaliana that can be used to generate large data sets amenable to interference analysis quickly. A. thaliana quartet (qrt) mutants produce pollen tetrads in which the four meiotic products are held together, allowing all products of a single meiotic event to be studied relative to one another. This assay system is based on a collection of transgenic lines, in the qrt background, each carrying a gene encoding either a red, cyan or yellow fluorescent protein that can be excited by different wavelengths of light. Expression of these markers is directed by a postmeiotic pollen-specific promoter (LAT52). Researchers can construct visually assayable genetic intervals by crossing lines that carry linked markers. Lines carrying two or more marker genes on the same chromosome expressing differently colored proteins produce tetrads that segregate the marker genes (and thus the proteins they encode) in the pollen tetrads in patterns that reflect whether or not a recombination event has happened between them. This system, can detect CO events directly in the pollen grains, and through the construction of double intervals delineated by three colors, can be used to measure CO interference throughout the A. thaliana genome.

We have constructed a genome-wide library of single-insertion fluorescent-tagged lines (FTLs) by transforming A. thaliana (Col) with these marker genes. All the markers shown in Figure 1 map outside genes. There are currently 113 FTLs available including 35 DsRed2, 41 eYFP and 37 eCFP. Combinations of these three groups can be crossed to create lines with three different markers. Pollen from plants that are heterozygous for these markers is viewed using an epi-fluorescence microscope with three different filters. Such a set of three linked markers on chromosome 5 has been used by our lab to detect subtle differences in interference between wild-type and mus81 mutant plants.

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**Figure 1 | Map of fluorescent transgenes.** The chromosomal (green bars) insertion site of the transgene carried by each fluorescent-tagged line (FTL) is indicated by a red (DsRed2), yellow (eYFP) or cyan (eCFP) circle. The identification number of each insertion is given above the circle. The genetic intervals (Ia, Ib, Ic, Id, Ie) that are available by request from G.P.C. are delineated by brackets. The Arabidopsis Information Resource (TAIR) ‘chromosome map tool’ (http://www.arabidopsis.org/) was used to place T-DNA insertion points on the map. T-DNA, transferred DNA.
Advantages and limitations of FTL interference analysis

The FTL system is capable of generating large data sets rapidly. An experienced student can score ~ 500 three-color tetrads in an hour. In addition, the FTL system is flexible in that it is possible to assay interference in almost any genomic location using the extensive collection of marker lines. These characteristics make comparative experiments designed to detect differences in interference levels between sample populations, such as mutants or experimental treatments, routinely feasible.

Although it is possible to score interference in most genomic locations, some sections of the genome have few or no mapped FTL insertions. An insert of each color is necessary in any particular region, and some regions do not meet this requirement. Markers in regions near centromeres are particularly sparse, and chromosomes 2 and 4 do not have the marker density of chromosomes 1, 3 and 5 (see Fig. 1). In addition, the FTL system produces data only from male recombination, and in A. thaliana, male and female CO levels, as well as interference, are significantly different14,28.

Meiotic-recombination mutants often exhibit lower levels of pollen viability when compared with wild type due to inaccurate chromosome segregation or chromosome fragmentation. Chromosomes that experience an aberrant number of exchanges are more likely to segregate improperly and thus produce inviable products, so the viable products of a mutant meiosis can be enriched for COs compared with inviable products. At present, the FTL system can be used only to assay COs in viable pollen grains, so it is possible that when analyzing mutant tetrads, the total change in COs can be underestimated. However, in an example in which a mutant (Atmsh4) has been analyzed by both the FTL and cytological methods (which are not thought to artificially enrich for COs), the CO data were very similar and the conclusions also similar17,31.

Using this system, it is possible to analyze two intervals simultaneously. Available genetic methods in some organisms allow the researcher to analyze simultaneously as many intervals as they have distinguishable markers, so these other methods may be better for whole-chromosome views of interference. Drouaud et al. recently monitored the segregation of 44 markers on a single A. thaliana chromosome and used a sliding window analysis system to analyze sex-specific interference levels on a single chromosome14. This type of analysis is not currently possible using the FTL system.

Applications of the fluorescent tetrad system

Other than assaying CO interference in adjacent intervals, this system can be used to assay CO rates in single intervals (which is a requisite for interference analysis in this system). The constructs created in this protocol can also be used in a visual assay for pollen viability.

Analysis of CO interference

Several methods have been described for analyzing interference19,25,44–46, but here, we will use the method described by Malkova et al., to measure interference using pollen tetrad data. In brief, the Malkova et al. method compares the map distance of one interval when an adjacent interval has no CO to the map distance of the first interval when the adjacent interval does have a CO19. If the genetic distance in the interval in question is significantly lower with the presence of a CO in the adjacent interval, one can conclude interference extends from one interval to the other. The ratio of distance (without adjacent CO/with adjacent CO) gives a measure of interference. A value of 1 indicates no interference; a value of 1 indicates complete interference and values between 1 and 0 correlate with the strength of interference.

Map distances can be calculated using the Perkins mapping function: $X = 100[(1/2T + 3NPD)/n]$ (ref. 47). This equation calculates an approximation of map distance in a single interval, but its validity diminishes for intervals that sometimes contain more than two COs. For this equation, tetrads are designated as PD, NPD or tetratype (T), depending on the segregation of the marker pair defining each interval.

The Malkova et al. method can also be used to compare interference data from two data sets (e.g., wild type and mutant) from the same intervals. One first calculates the ratio of map distance without adjacent CO/map distance with adjacent CO as above for each data set. These ratios can be statistically compared by obtaining a Z-score using the following equation:

$$Z = \frac{|R_1 - R_2|}{\sqrt{\text{var}(R_1 - R_2)}}$$

where $R_1$ is the ratio in treatment 1 (e.g., wild type) and $R_2$ is the ratio in treatment 2 (e.g., mutant).

The significance of the difference between these two ratios can be assessed using a one-tailed test as described on the Stahl Lab Online Tools (http://molbio.uoregon.edu/~fstahl).

When working with tetrad data, it is also possible to calculate interference using a single interval25. One can estimate the fraction of NPD tetrads expected in the case of no interference from the fraction of T tetrads observed in an interval using the formula developed by Papazian25:

$$\text{Fraction of NPDs expected} = 0.5 \left[ (1 - fT) - \left( 1 - \left( \frac{3fT}{2} \right) \right)^{n/2} \right]$$

where $fT$ is the fraction of tetratypes observed.

As in the methods described above, one can then divide the observed fraction of NPDs by the expected to get a measurement of the strength of interference in this interval. The Papazian method, relying as it does on just the T frequency to calculate expected NPDs, uses data inefficiently. A method for detecting interference that uses tetrad data more efficiently is available online at Stahl Lab Online Tools (http://molbio.uoregon.edu/~fstahl).

Interference can also be measured directly from meiotic chromosomes that have been appropriately labeled such that the chromosomes are distinguishable from one another and CO sites are visible13,35. Inter-CO distances can be measured using a computer software such as NIH image (http://rsb.info.nih.gov/nih-image/). The distribution of these distances can be fit to gamma distributions that simulate CO placements with varying degrees of interference. The best fit distribution can be used to estimate the interference parameter $\phi$11,32,33,48.

In this protocol, we describe a method for using the FTL system to generate large data sets amenable to CO interference analysis quickly in the flowering plant A. thaliana. We describe how to create adjacent intervals by crossing marker-insertion lines from the FTL library and how to visually screen and score these intervals for COs.
**PROCEDURE**

**Selection of appropriate FTLs**

1) From the FTL insert library, select the appropriate lines that will define two adjacent intervals (5 cM per Mb is a reasonable estimate for the average genetic-to-physical ratio in *A. thaliana*).

**Critical Step** One line for each of the eCFP, DsRed2 and eYFP markers needs to be selected, but they may occur in any order. The sizes of the two intervals do not have to be similar, but it will slightly complicate analysis if either of the intervals is large enough such that an abundance of NPD tetrads in either single interval is observed. Note that for the remainder of the protocol, the line that carries an insert closest to the North end of the chromosome will be referred to as ‘A’, the next will be ‘B’ and the line with an insert closest to the South end will be ‘C’.

**Construction of first interval (I1)**

2) Plant at least 12 seeds from line A and line B (seeds will usually be heterozygous for the interval). Grow plants on Metro-mix 400 soil in an environmentally controlled growth chamber with a long photoperiod (16 h light at 20 °C/8 h dark at 20 °C).

**Critical Step** Steps 2–11 can be omitted if the researcher wishes to use interference intervals that our lab has already constructed (see Fig. 1). These lines will be supplied as a segregating population of seeds from an ABC/+++ parent and are available by request from G.P.C.

**Critical Step** If the study includes analysis of interference in mutant backgrounds, it will save time to cross the mutants into the qrt1 background during the construction of the three-color interval. We recommend maintaining and crossing meiotic mutants in the heterozygous state to avoid genomic abnormalities. Useful plants will have the genotype mutant/++; qrt1/qrt1.

3) As each marker will be segregating 1:2:1 in this generation, it is necessary to determine the marker genotype of each plant by examining the pollen under the epi-fluorescence microscope when plants begin flowering (4–5 weeks). As this step is diagnostic and does not require any counting, use the simplified screening process outlined in Box 1. Plants homozygous (4:0 fluorescent pollen, see Fig. 2) for their respective markers will be used for the next crossing step. Collect seeds from 2:2 plants (A/+ and B+/+) and 4:0 plants (A/A and B/B) to save as stocks.

**Pause Point** Store the seeds at room temperature (22–24 °C) in a labeled 1.5-ml polypropylene tubes with a small hole punctured at the top with a needle to prevent molding.

4) To create the first interval, cross a line-A plant that is homozygous for one color insert to a line-B plant homozygous for a different colored insert. Make several crosses to be sure of a viable cross. For details and tips on crossing *A. thaliana*, see ref. 49. The stigma of the female used for the cross will mature into a silique containing seeds used for the next step.

**Troubleshooting**

5) After the siliques from the crosses (Step 4) have dried (2–3 weeks), harvest the seeds and plant them as described in Step 2. At the same time, plant at least 12 seeds from line C, which will define the second interval.

6) When these plants begin flowering (4–5 weeks), diagnose the fluorescent genotypes of each plant as described in Box 1. Individuals from the A–B cross should yield pollen tetrads with the same fluorescent pattern (2 color-A:2 color-B); discard plants that do not. For line C, select plants homozygous for the C marker. Save seed stocks from the A/B plants and the C/+ and C/C plants as described in Step 3.
BOX 1 | SIMPLIFIED SCREENING PROCESS

1. Rub a microscope slide briskly for ~10 s with a soft cotton cloth. This is necessary so that the PGM holds its drop formation.
2. Drop six evenly spaced 10-μl spots of PGM onto the slide.
3. Using forceps remove an open flower and place it face down in the first drop of PGM. Let it soak for 20 s.
4. Use a gentle tapping motion to release the pollen into the PGM. This is best done on a black bench top that will allow the researcher to see the pollen being released into the solution.
5. Repeat Steps 3 and 4 for five more flowers until the slide is full.
   ▲ CRITICAL STEP Work quickly so that the drops of PGM do not dry out before screening. Drops that dry out can be rehydrated by adding a second drop of PGM.
6. View and diagnose the pollen under the appropriate filter using the epi-fluorescence microscope. If a plant is homozygous for the marker, all four pollen grains in each tetrad will be fluorescent. If a plant is heterozygous for the marker, each tetrad will fluoresce in a 2:2 fluorescent:nonfluorescent pattern. Likewise, if the plant is homozygous wild type, no pollen will fluoresce (Fig. 2).

Construction of second interval (I2), and cross into mutant background
7| CRITICAL STEP This cross needs to be made several times because the useful plants in the next generation will have all the three markers, which can only be achieved when C is fertilized with a recombinant pollen grain harboring both the A and B inserts in a cis configuration. The probability (as a percent) of this event can be calculated by dividing the recombinant frequency between A and B (in percent) by 2. This should give a good idea of how many crosses to complete (depending on how many seeds you usually get from your crosses). As a general rule, perform enough crosses to ensure that you have three times as many seeds as would be needed to produce a single three-colored plant.

? TROUBLESHOOTING
8| After the siliques from these crosses have dried (2–3 weeks), plant all of the seeds as described in Step 2. Also plant at least eight qrt1/qrt1 (nonfluorescent) seeds and/or the mutant/+; qrt1/qrt1 seeds.

9| When these plants begin flowering, diagnose the marker genotype of each plant as described in Box 1. Each of the three fluorescent marker genotypes needs to be determined under the appropriate colored filter in succession. Pollen tetrads from useful plants will fluoresce 2AB:2C, having the genotype AB+/++C. The markers will have had a chance to recombine during the meiosis that produced the tetrads being examined. In useful plants, the tetrads will show many combinations in relation to one another. What is important is that all the three colors show a 2:2 pattern.

10| At this stage, if the study includes interference analysis in mutant backgrounds, cross the three color lines to mutant/+; qrt1/qrt1 lines. Otherwise, cross the three color lines to the Col qrt1 mutants using a similar strategy as in Step 7 with the purpose of producing lines that are ABC/+++; qrt1/qrt1 (and optionally mutant/+).

? TROUBLESHOOTING
11| When the siliques from these crosses (Step 10) have dried (2–3 weeks), plant all of the seeds. Useful plants will have the genotype ABC/+++ and show a majority of pollen tetrads with a 2ABC: 2 nonfluorescent pattern. These plants can be analyzed for interference, but in order to generate large amounts of data from multiple individuals, it is necessary to analyze the appropriate progeny from self-crosses. If crosses were made into mutant backgrounds, useful plants will have the genotype ABC/+++; mutant/+; qrt1/qrt1.

Scoring three-color tetrads for recombination
12| Plant at least 50 seeds collected from ABC/+++; (mutant/+); qrt1/qrt1 individuals. These plants need to be distinguished from one another throughout the scoring process, so it is useful to sow the seeds in divided flats. We plant in 6 × 4 divided flats (24 cells) where the flats measure 26 × 54 cm² and each cell measures 6.5 × 9 cm². This provides each plant enough room to grow while it is also easy to distinguish one plant from another. At this stage, the plants need to be grown in a temperature-controlled growth chamber.

Figure 2 | Single locus segregation patterns in pollen tetrads. A locus with a transgenic marker construct encoding a fluorescent protein (in this case DsRed2) can be homozygous (A/A) or hemizygous (A+/) for the marker or it can be wild type (+/+). The fluorescence signal in the pollen tetrads will reflect the marker genotype and yield 4:0 (left) 2:2 (middle) or 0:4 (right) pollen tetrads respectively.
**CRITICAL STEP** Columbia FTLs have been extensively tested in our lab. In general, *A. thaliana* plants are sensitive to environmental changes, so it is important to keep a constant environment as much as possible. Work done in our lab has shown that temperature levels influence recombination, so it is critical to maintain the same levels for all experimental and control populations\(^\text{16}\). 

| CRITICAL STEP | After the plants begin flowering (4–5 weeks), it is necessary to determine the fluorescent genotype of each individual plant as described in Box 1. Only plants that are ABC/+++ can be scored for recombination in both intervals simultaneously; discard all others. Note that plants of the genotype ABC/ABC are useful for quantifying pollen viability, see Box 2 for details\(^\text{17}\). |
| CRITICAL STEP | To begin the scoring process, select an ABC/+++ plant for analysis. |
| CRITICAL STEP | Rub a microscope slide briskly for ~10 s with a soft cotton cloth. |
| CRITICAL STEP | Place two 10-μl drops of PGM onto the slide ~3 cm apart. The drops should form a tight bead on the slide. |
| CRITICAL STEP | Using the forceps, remove a single open flower and place it face down into the first drop of PGM, allow to soak for at least 20 s. Note the flower number on the plant by counting up the bolt. |
| CRITICAL STEP | Use a tapping motion to release the pollen into the PGM. This is best done on a black bench top that will allow the researcher to see the pollen being released into the solution. Remove the flower from the first drop of PGM and repeat the process with the same flower in the second drop. This maximizes the amount of pollen that can be scored from each flower. Gently place a cover slip on top of each pollen-containing drop of PGM. |
| CRITICAL STEP | Screen the tetrads on the ×10 objective. A pollen grain is classified as dead (inviable) if it is either (i) shrunken up/aborted or (ii) fully developed, but all of the fluorescent markers fail to express in the grain (i.e., it appears blank). In any given tetrad, you may observe a combination of live/dead grains. Depending on the pattern of dead-to-alive grains, each tetrad can be classified as 4:0, 3:1, 2:2, 1:3, 0:4 (ref. 17). Pollen viability can be quantified as a percent of dead grains/total grains, and the percentages of each class of tetrad can be presented in a histogram. Many meiotic mutants show a characteristic elevation in the 2:2 class as compared with wild type, which typically shows a total viability of >95%. |

**TABLE 1** | Letter codes for classification of tetrads. |
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DCO, double crossover; NPD, nonparental dittype. *st = strand.
North–South order of the different colored markers will determine how the fluorescent tetrads are categorized. To determine how to classify each type of tetrad fluorescence pattern, it may be useful to redraw Figure 3 using the appropriate configuration of markers (Supplementary Fig. 1 online).

▲ CRITICAL STEP Do not score tetrads in clumps, as this can lead to erroneously classifying tetrads. The Triton-X detergent is added to the PGM to prevent clumping. Only score four-member tetrads.

20| If a mutant allele is segregating in this generation, genotype the mutants after scoring is complete. Blind scoring is a good experimental practice to avoid bias in the counting process.

21| Repeat for as many plants as the analysis demands—this will differ from experiment to experiment.

• TIMING
Steps 1–6, selection of appropriate FTLs and construction of the first interval: 6–7 weeks
Steps 7–11, construction of the second interval, and cross into mutant background: 12–14 weeks
Steps 12–21, scoring three-color tetrads for recombination: 4–6 weeks

### TABLE 2 | Example of data produced by one individual plant.

| Plant | Screen date | Flower | NR | SCO-I1 (R/Y) | SCO-I2 (Y/C) | 2 st* | 3 st* | 3 st* | 4 st* | DCO | DCOa | DCOb | NPD-I1 | NCO-I1 | NPD-I2 | NCO-I2 | NPD-I1 | NPD-I2 | NPD-I1 | NPD-I2 | Total |
|-------|-------------|--------|----|--------------|--------------|-------|-------|-------|-------|-----|-------|-------|--------|--------|--------|--------|--------|--------|--------|-------|
| 2B2   | 7Mar        | 4      | 54 | 45           | 25           | 2     | 2     | 3     | 0     | 0   | 0     | 0     | 0      | 0      | 0      | 0      | 0      | 0      | 131    |
| 9Mar  | 11          | 49     | 38 | 19           | 0            | 1     | 2     | 1     | 1     | 1   | 0     | 0      | 0      | 0      | 0      | 0      | 0      | 112    |
| 9Mar  | 12          | 35     | 29 | 20           | 2            | 0     | 3     | 0     | 0     | 0   | 0     | 0      | 0      | 0      | 0      | 0      | 0      | 89     |
| 12Mar | 21          | 41     | 54 | 23           | 2            | 2     | 2     | 4     | 3     | 0   | 1     | 0      | 0      | 0      | 0      | 0      | 0      | 130    |
| 12Mar | 22          | 28     | 32 | 32           | 1            | 0     | 3     | 0     | 3     | 0   | 0     | 0      | 0      | 0      | 0      | 0      | 0      | 99     |
| Totals|             |        |    | 207          | 198          | 119   | 7     | 5     | 15    | 4   | 4     | 2     | 0      | 0      | 0      | 0      | 0      | 561    |

DCO, double crossover; NPD, nonparental ditype. *st = strand.

### TABLE 3 | Example of aggregate data comparing a mutant to wild type.

<table>
<thead>
<tr>
<th>NR</th>
<th>SCO-I1 (R/Y)</th>
<th>SCO-I2 (Y/C)</th>
<th>2st DCO</th>
<th>3st DCOa</th>
<th>3st DCOb</th>
<th>4st DCO</th>
<th>NPD-I1 NCO-I1</th>
<th>NPD-I2 NCO-I2</th>
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<td>3065</td>
<td>1712</td>
<td>110</td>
<td>112</td>
<td>89</td>
<td>103</td>
<td>62</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>--/+/--</td>
</tr>
</tbody>
</table>

DCO, double crossover; NPD, nonparental ditype.
**TROUBLESHOOTING**

Troubleshooting advice can be found in Table 4.

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4, 7, 10</td>
<td>Inviable crosses, crosses not producing many seeds</td>
<td>Stigma damaged during emasculation, plants too old</td>
<td>Use new forceps that are sharp. Use a jeweler’s magnifying glass. Emasculate young buds that are as large as possible</td>
</tr>
<tr>
<td>18</td>
<td>Few scorable tetrads</td>
<td>Tetrads broken apart, few tetrads released into PGM, mutant causes lethality</td>
<td>Be gentle when tapping pollen out of flower. Cover slip should be dropped gently, never squashed. It may help to let flower soak in PGM for &gt;20 s. Some mutants do not produce a lot of viable tetrads, and we find that it helps to score these mutants on lower magnification (×10) so that viable tetrads can be identified more quickly. Most mutants produce some viable tetrads, so low viability can be overcome by scoring a lot of flowers from these individuals</td>
</tr>
</tbody>
</table>

**ANTICIPATED RESULTS**

The type of results for a single plant generated by three-color FTL interference experiments is shown in Table 2. Compiled results from an experiment designed to test the interference phenotype of a mutant is shown in Table 3. If the object of the analysis is to test a mutant phenotype, and the crosses are conducted in the manner described, then the mutant (assuming recessivity) will be segregating 1:3 in the scoring generation. This is ideal because the blind controls for the experiment are built into the design.

To calculate interference levels from the data that are produced using this method, we recommend using the Malkova et al. method for quantifying interference19. Using this method, the researcher determines the map distance of one of the intervals without and with a CO in the adjacent interval, and the ratio between the two represents the level of interference. Using letter codes from Table 1, the following formulas are used:

\[
X_{I1}(w/o \text{adjacent } CO) = \frac{\left( \frac{1}{2}T + 3\text{NPD} \right)}{\text{total}} = \frac{\left( \frac{1}{2}B \right) + 3(H)}{(A + B + H)}
\]

\[
X_{I1}(\text{with adjacent } CO) = \frac{\left( \frac{1}{2}T + 3\text{NPD} \right)}{\text{total}} = \frac{\left( \frac{1}{2}(D + E + F + G + K) + 3(J + L) \right)}{(C + D + E + F + G + I + J + K + L)}
\]

Interference ratio = \( X_{I1} \) w/o adjacent CO/\( X_{I1} \) with adjacent CO, where, as above, \( X \) refers to the map distance generated from the Perkins equation44.

Ratios between sample populations can be compared using Stahl Lab Online Tools: http://molbio.uoregon.edu/~fstahl.

**Examples of interference analysis using data generated by the FTL system**

From an individual plant (Table 2):

\[
X_{I1}(w/o \text{adjacent } CO) = \frac{\left( \frac{1}{2}B \right) + 3(H)}{(A + B + H)} = \frac{\left( \frac{1}{2}(198) + 3(4) \right)}{(207+198+4)} = 27.1 \text{ cM}
\]

**Figure 4** | Examples of multicolor fluorescent tetrads. To assess three-color intervals each tetrad must be visualized through each of three different fluorescent filters (red, yellow and cyan) on the epi-fluorescence microscope. These individual images can be merged (right column) into a composite image using graphics software such as Adobe Photoshop. A plant that is heterozygous for three markers in cis configuration can yield nonrecombinant pollen tetrads that have all the three colors in the same two pollen grains (top row). A single crossover (CO) (in I1 in this case) will yield a pollen tetrad that has one grain with all three colors, one grain with two colors, one grain with one color and one with no color (middle row). Double COs (DCOs) will yield pollen tetrads with a variety of segregation patterns (see Fig. 3), in this case a four-strand DCO results in a tetrad with one red, one yellow/blue, one blue and one red/yellow grain (see Fig. 3g).
$X_{11}(\text{with adjacent CO}) = \frac{(1/2(D+E+F+G+K)+3(J+L))}{(C+D+E+F+G+I+J+K+L)} = \frac{(1/2(7+5+15+4+0)+3(0+0))}{(119+7+5+15+4+2+0+0+0)} = 10.2 \text{ cM}$

Interference ratio $= X_{11} \text{ w/o adjacent CO} / X_{11} \text{ with adjacent CO} = \frac{10.2}{27.1} = 0.38$

Comparing compiled data from two sample populations (Table 3):

$+/+$ and $+/-$ (row 1)

$X_{11}(\text{w/o adjacent CO}) = \frac{((1/2B)+3(H))}{(A+B+H)} = \frac{((1/2(3881))+3(68))}{(3729+3881+68)} = 27.9 \text{ cM}$

$X_{11}(\text{with adjacent CO}) = \frac{(1/2(D+E+F+G+K)+3(J+L))}{(C+D+E+F+G+I+J+K+L)} = \frac{(1/2(128+161+149+129+6)+3(9+0))}{(2286+128+161+149+129+21+9+6+0)} = 10.9 \text{ cM}$

Interference ratio

Interference ratio $= X_{11} \text{ w/o adjacent CO} / X_{11} \text{ with adjacent CO} = 0.3885$

$-/–$ mutant (row 2)

$X_{11}(\text{w/o adjacent CO}) = \frac{((1/2B)+3(H))}{(A+B+H)} = \frac{((1/2(3065))+3(62))}{(2785+3065+62)} = 29.1 \text{ cM}$

$X_{11}(\text{with adjacent CO}) = \frac{(1/2(D+E+F+G+K)+3(J+L))}{(C+D+E+F+G+I+J+K+L)} = \frac{(1/2(110+112+89+103+1)+3(1+0))}{(1712+110+112+89+103+16+1+1+0)} = 9.8 \text{ cM}$

Interference ratio $= X_{11} \text{ w/o adjacent CO} / X_{11} \text{ with adjacent CO} = 0.3378$

Test of significance between two ratios of map distances (Stahl Lab Online Tools)
The following data were input:

Interval name $= \text{wt with adjacent CO}$
PD $= A = 3,729$; T $= B = 3,881$; NPD $= H = 68$

Interval name $= \text{wt without adjacent CO}$
PD $= C + I = 2,307$; T $= D + E + F + G + K = 573$; NPD $= J + L = 9$

Interval name $= \text{mutant with adjacent CO}$
PD $= A = 2,785$; T $= B = 3,065$; NPD $= H = 62$

Interval name $= \text{mutant without adjacent CO}$
PD $= C + I = 1,728$; T $= D + E + F + G + K = 415$; NPD $= J + L = 1$

The program gives this output:
Ratio definitions $R1 = \text{wt with/wt without}$
Ratios $R1 = 0.3885$ $R2 = 0.3378$
Variance of ratios $\text{var}R1 = 0.00032444$ $\text{var}R2 = 0.00026894$
Standard error of ratios $\text{S.E.} R1 = 0.0180122$ $\text{S.E.} R2 = 0.01639943$
$\text{Var}[R1 – R2] = 0.00059338$
$|R1 – R2| = 0.05075571$

(One tailed) Is $|R1 – R2| > 1.65 \times \sqrt{\text{Var}[R1 – R2]}$? $0.0508 > 0.0402$ significant

In this case, the mutant ratio was significantly different from the wild-type ratio and we concluded that the two genotypes had different interference levels. The mutant has significantly stronger interference than the wild type.
PROTOCOL

Note: Supplementary Information is available via the HTML version of this article.

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