Formamide-Free Genomic in situ Hybridization Allows Unambiguous Discrimination of Highly Similar Parental Genomes in Diploid Hybrids and Allopolyploids

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Key Words

Allopolyploid · Formamide-free GISH · Hybrid · *Melampodium* · *Prospero* · Standard GISH protocol

Abstract

Polyploidy and hybridization play an important role in plant diversification and speciation. The application of genomic in situ hybridization (GISH) allows the identification of parental genomes in hybrids, thus elucidating their origins and allowing for analysis of their genomic evolution. The performance of GISH depends on the similarity of the parental genomes and on the age of hybrids. Here, we present the formamide-free GISH (ff-GISH) protocol applied to diploid and polyploid hybrids of monocots (*Prospero*, Hyacinthaceae) and dicots (*Melampodium*, Asteraceae) differing in similarity of the parental genomes and in chromosome and genome sizes. The efficiency of the new protocol is compared to the standard GISH protocol. As a result, ff-GISH allowed efficient labeling and discrimination of the parental chromosome sets in diploid and allopolyploid hybrids in *Prospero autumnale* species complex. In contrast, the standard GISH protocol failed to differentiate the parental genomes due to high levels of similar repetitive DNA. Likewise, an unambiguous identification

of parental genomes in allotetraploid *Melampodium nayaritense* (Asteraceae) was possible after ff-GISH, whereas the standard GISH hybridization performance was suboptimal. The modified method is simple and non-toxic and allows the discrimination of very similar parental genomes in hybrids. This method lends itself to modifications and improvements and can also be used for FISH.

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Polyploidy and hybridization play an important role in plant diversification and speciation [Soltis and Soltis, 2009]. The major challenge in studying hybrids at both the diploid and polyploid level is the unambiguous identification of parental taxa, which subsequently contributes to in-depth analyses of the evolution of polyploid genomes. Various methods are employed in this context, including morphological analyses, phylogenetic analyses of nuclear and plastid DNA markers, fingerprinting methods, and cytogenetics [Lihová et al., 2007; Weiss-Schneeweiss et al., 2012]. The latter encompasses classical and molecular methods, most notably fluorescence in situ hybridization (FISH) and genomic in situ hybridization (GISH). In particular, GISH allows differential labeling of

Fig. 1. Schematic showing the representation of genome evolution in the analyzed taxa of plant genera *Prospero* (A) and *Melampodium* (B), respectively.

the parental genomes in hybrids. The success rate of GISH, however, strongly depends on the similarity of the parental genomes (the more similar, the more difficult it becomes to distinguish the parental genomes) and on the evolutionary age of the hybrids (the older the hybrids, the more difficult it is to distinguish the parental genomes) [Lim et al., 2005]. Since its first application [Schwarzacher et al., 1989], various GISH protocols have been developed to successfully differentiate parental genomes in plants [Choi et al., 2008; Pendinen et al., 2008; Mandáková et al., 2014]. However, most of these are optimized specifically for certain plant groups [Renny-Byfield et al., 2010; Jeridi et al., 2011; Chester et al., 2012, 2015]. The major limitation of the use of GISH in plant hybrids is poor performance when closely related parental genomes (species or varieties) which share the majority of the repetitive DNA elements are involved in hybridization.

Diploid and polyploid hybrids of 2 plant groups, representing monocots and dicots, of known parental origin were analyzed in the current study (fig. 1) [Weiss-Schneeweiss et al., 2012; Jang et al., 2013]. Specifically, diploid homoploid and polyploid hybrids of the monocot *Prospero autumnale* complex (Hyacinthaceae) with relatively large genome sizes, and polyploid hybrids of the dicotyledonous genus *Melampodium* (Asteraceae) with small ge-

nome sizes were targeted. The circum-Mediterranean *P. autumnale* complex, 1 of the 3 species in the genus, is cytologically variable [Jang et al., 2013]. The 4 diploid cytotypes of this complex differ in the combination of their basic chromosome number and genome size [Jang et al., 2013]. Cytotypes AA and B⁷B⁷ [Vaughan et al., 1997; Jang et al., 2013] possess a basic chromosome number of $x = 7$ but differ in genome size, with cytotype AA having a 70% larger genome than B⁷B⁷ (1C of 7.85 pg and 4.23/4.45 pg, respectively) [Jang et al., 2013]. Cytotypes B⁶B⁶, endemic to Crete, and B⁵B⁵, endemic to Libya, underwent independent reductions of base chromosome numbers to $x = 6$ and $x = 5$, respectively, as well as increases of genome sizes (1C of 6.27 pg and 4.68 pg, respectively) [Jang et al., 2013]. Analyses of the repetitive DNA fraction of all 4 diploid cytotypes revealed the presence of one widespread, but variable in copy number, satellite DNA *PaB6* [Emadzade et al., 2014], and similar relative proportions of all major lineages of transposable elements [Weiss-Schneeweiss et al., unpubl. data]. Although the 4 cytotypes form phylogenetically distinct groups [Jang et al., 2013], they are nearly morphologically indistinguishable. Almost all diploid cytotypes, except for the B⁵ genome, form polyploids, allopolyploids (combinations of A and B⁷ as well as B⁶ and B⁷ genomes), and autopolyploids originating from the B⁷ genome [Vaughan et al., 1997]. In addition, all diploid cytotypes can be crossed and produce viable diploid homoploid hybrids [Taylor, 1997; Jang et al., 2013].

The dicotyledonous genus *Melampodium* (Asteraceae), endemic to Mexico and adjacent territories, encompasses 40 species in 8 sections [Stuessy et al., 2011]. Five different basic chromosome numbers occur in diploids of this genus, $x = 9, 10, 11, 12$, and 14 [Weiss-Schneeweiss et al., 2009]. The largest section, *Melampodium*, comprises 20 species grouped into 5 series, all with a base chromosome number of $x = 10$. One of the series of this section, series *Sericea*, contains exclusively tetraploid and hexaploid species, all of hybrid origin. The parental origins of these allopolyploids were inferred using phylogenetic analyses of nrITS, 5S rDNA spacer (NTS), plastid regions, and the low copy gene *PgiC*, and general trends in rDNA evolution were inferred via FISH [Blösch et al., 2009; Weiss-Schneeweiss et al., 2009, 2012; Stuessy et al., 2011]. The diploid species *M. linearilobum* and *M. longipes* (although its sister taxon *M. americanum* could not be excluded) were identified as the parental species of the allotetraploid *M. nayaritense* [Weiss-Schneeweiss et al., 2012]. The 2 diploids share the same base chromosome number of $x = 10$, but differ in genome size (1C of 0.49 pg vs. 1.12 pg, respectively) [Weiss-Schneeweiss et al., 2012].

and consequently in the proportion and the composition of the repetitive DNA sequences [McCann et al., unpubl. data].

The aim of this study was to develop a simple GISH protocol that could be used across various plant groups and would effectively distinguish parental genomes in hybrids regardless of their parental genome similarity, genome size, repeat composition, and taxonomic affiliations. Most available protocols include organic solvents, most commonly formamide, in hybridization mixes which lowers the effective DNA melting temperature and allows hybridization at lower temperatures without compromising the stringency [Meinkoth and Wahl, 1984]. Formamide, however, prolongs the time of hybridization by slowing down the rate of renaturation [Berndt et al., 1996; Blake and Delcourt, 1996]. The performance of newly established formamide-free GISH protocol (from now on called ff-GISH) in the 2 above-mentioned plant groups was compared to the commonly used formamide-based GISH protocol [Schwarzacher and Heslop-Harrison, 2000].

Materials and Methods

Plant Material

P. autumnale Complex. A diploid homoploid hybrid of the genomic composition B⁶B⁷ (F₁ cross between diploid cytotypes B⁶B⁶ and B⁷B⁷; H364, Crete, Greece) and an allopolyploid hybrid AAB⁷B⁷ (H603, Algarve, Spain) were used for analyses (fig. 1A). Plants were cultivated in the Botanical Garden of the University of Vienna (HBV).

Melampodium. Allotetraploid *M. nayaritense* (M115, Nayarit, Mexico) was analyzed (fig. 1B). Seeds were collected from natural populations in Mexico in August 2013 and germinated, and plantlets were cultivated in the Botanical Garden of the University of Vienna (HBV).

Actively growing root meristems of all plants were harvested, pretreated with colchicine (for *Prospero*) [Jang et al., 2013] or 8-hydroxyquinoline (for *Melampodium*) [Weiss-Schneeweiss et al., 2009], fixed in 3:1 ethanol:acetic acid mixture, and stored at -20°C until use.

Genomic in situ Hybridization

Enzymatic digestion of fixed root meristems and chromosome preparations were made as described earlier [Weiss-Schneeweiss et al., 2012; Jang et al., 2013]. GISH was carried out following standard chromosome pretreatment with RNase and pepsin [Weiss-Schneeweiss et al., 2012].

DNAs of putative parental genomes were used as probes (fig. 1). Total genomic DNAs from *P. autumnale* diploid cytotypes AA, B⁶B⁶, and B⁷B⁷ as well as *M. linearilobum* (2x) and *M. longipes* (2x) were isolated using the CTAB method [Doyle and Doyle, 1987; Jang et al., 2013] and sheared at 98°C for 5 min. Approximately 1 µg of genomic DNA of each cytotype was labeled with either di-

goxigenin or biotin using a nick translation kit (Roche, Vienna, Austria). The fragment length of the resulting probes was about 100 bp. Probes were purified via ethanol precipitation and resuspended in water.

The GISH mix consisted of 10% dextran sulfate (Sigma Aldrich, Vienna, Austria), 0.02×SSC, 1% salmon sperm DNA, and 3–4 ng/µl of each of 2 labeled genomic DNA probes. The hybridization mix was denatured at 98°C for 5 min and stored at 4°C. Hybridization mix (10 µl per slide containing 30–40 ng of each of the labeled genomic probes) was applied to each slide and sealed under a coverslip. Combined denaturation of the material on the slide and hybridization mix was performed at 72°C for 4 min, and they were allowed gradually to cool down to 37°C (1 min at 65°C, 1 min at 55°C, and 1 min at 45°C) on a PCR in situ block. Hybridization was carried out for 12–24 h at 37°C. Stringent washes were performed 3 times in 2×SSC at 42°C for 3 min each. Slides were incubated for 20 min at 37°C in blocking solution containing 5% bovine serum albumin (BSA) and Tween20 in 2×SSC buffer. Probes labeled with digoxigenin were detected using 1 ng/µl FITC-conjugated antidigoxigenin (Roche), and biotin was detected using 3 ng/µl Cy3-conjugated ExtraAvidin (Sigma), both in 2×SSC containing 5% (w/v) BSA and 0.5% Tween20 for 1 h at 37°C. Subsequently, preparations were washed twice in 2×SSC and once in 2×SSC, 0.5% Tween20 at 42°C, 3 min each. DNA was counterstained with 2 ng/µl DAPI (4',6-diamidino-2-phenylindole) and mounted in Vectashield antifade medium (Vector Laboratories, Burlingame, Calif., USA). Preparations were analyzed with an AxioImager M2 epifluorescent microscope (Carl Zeiss, Vienna, Austria), and images were captured with a CCD camera and processed using AxioVision ver. 4.8 (Carl Zeiss) using only those functions that apply equally to the entire image. A detailed step-by-step protocol is available as online supplementary material (online suppl. file 1; see www.karger.com/doi/10.1159/000441210 for all online suppl. material).

As a control, GISH using the standard hybridization mix [Schwarzacher and Heslop-Harrison, 2000] was performed in parallel. To test the effect of the formamide-free hybridization solution on the denaturation of the chromosomes, additional experiments were performed where denaturation of the preparations was performed in Coplin jars in 70% formamide solution and only then was the denatured hybridization mix added to the slides. This experiment was again performed for both standard and formamide-free hybridization mixes in parallel.

The performance of the present method has also been compared to another formamide-free GISH protocol applied earlier to *Tragopogon* chromosomes (Asteraceae) [Chester et al., 2012, 2015]. The hybridization mix composition and hybridization conditions from Chester et al. [2012] were used in this experiment whereas chromosome preparation, DNA labeling, and detection for practical and comparative reasons followed the ff-GISH protocol.

The *P. autumnale* hybrid B⁶B⁷ contained significant amounts of the tandem repeat *PaB6*, nearly all contributed by the B⁶ genome [Emadzade et al., 2014]. In order to reduce the effects of the strong hybridization of *PaB6* to pericentric chromosomal regions, which negatively affected image acquisition (signal-to-noise ratio), unlabeled monomers of the satellite DNA *PaB6* were added to the hybridization mix as blocking DNA in a concentration 20× higher than that of the labeled probes (~600–800 ng per slide). Again, this experiment was performed for both the standard mix and formamide-free hybridization mix.

Fig. 2. Comparison of standard formamide-containing (**B, E, H**) and formamide-free (**A, C, D, F, G, I**) GISH protocols in the plant genera *Melampodium* (Asteraceae) and *Prospero* (Hyacinthaceae). **A–C** Diploid homoploid hybrid B⁶B⁷ of *P. autumnale* complex. **D–F** Allotetraploid AAB⁷B⁷ of *P. autumnale* complex. **G–I** Allotetraploid *M. nayaritense*. **A, D, G** Denaturation and hybridization in formamide-free hybridization mix (ff-GISH¹). **C, F, I** Denaturation in 70% formamide and hybridization in formamide-free hybridization mix (ff-GISH²). Images **A** and **B-1** show result of hybridization using pericentric satellite *PaB6* as unlabeled blocking DNA. Image **B-2** shows hybridization without blocking satellite *PaB6*. Scale bars = 5 μm.

Results and Discussion

P. autumnale Complex

In *P. autumnale*, a diploid homoploid hybrid of B⁶B⁷ origin (fig. 2A–C) as well as an allotetraploid AAB⁷B⁷ (fig. 2D, F) were analyzed (fig. 1A). Only some of the individual chromosomes of the parental cytotypes could be identified by their distinct morphology or size, whereas a subset of chromosomes always remained unassigned in both hybrids. The standard GISH method did not allow efficient hybridization and discrimination of the parental chromosome sets, neither in the allopolyploid AAB⁷B⁷ (fig. 2E) nor in the diploid hybrid B⁶B⁷ (fig. 2B). This result was expected due to the high degree of similarity of repetitive DNA fractions of the individual cytotypes (over 90%) [Weiss-Schneeweiss et al., unpubl. data] despite quite significant genome size variation [Jang et al., 2013]. The new ff-GISH protocol, however, allowed unambiguous differentiation of the 2 parental chromosome sets (fig. 2A, D; online suppl. figs. 1, 2) both in the diploid and allotetraploid hybrid.

Satellite *PaB6* is highly amplified in the B⁶ genome but is nearly absent from the B⁷ genome [Emadzade et al., 2014], and its strong hybridization to pericentric regions of chromosomes of B⁶ origin in B⁶B⁷ hybrid genome hin-

ders acquisition of well contrasted images (fig. 2B-2). When GISH was performed only with 2 labeled genomic parental DNAs, satellite DNA signals after GISH were much stronger than any other signals. Addition of an excess of unlabeled, PCR-amplified monomers of *PaB6* to the hybridization mix reduced the intensity of most *PaB6* signals and allowed the acquisition of good quality GISH images (fig. 2A, B-1, C). The only prerequisite for such a procedure is the identification and isolation of most abundant satellite DNAs from the genomes of interest. It is not absolutely necessary for the success of ff-GISH procedure, but it certainly results in more optimal performance of the method.

Melampodium

The allotetraploid *M. nayaritense* (2n = 4x = 40) originated from a cross between diploid *M. longipes* (or less likely its sister species *M. americanum*) and diploid *M. linearilobum* (fig. 1B). Its karyotype is bimodal with 20 very small chromosomes contributed by *M. linearilobum* and 20 medium-sized chromosomes of *M. longipes* (fig. 2G–I). Due to this size difference, the parental chromosomes can easily be identified in the hybrid even in unstained preparations using phase contrast imaging, helping to verify the GISH performance.

GISH in allotetraploid *M. nayaritense* ($2n = 4x = 40$; fig. 2G–I) using the standard hybridization mix containing formamide (standard hybmix) [Schwarzacher and Heslop-Harrison, 2000] resulted in uneven chromosome labeling with low signal-to-noise ratio (fig. 2H). The small chromosomes of *M. linearilobum* origin were preferentially stronger labeled. Detailed analyses concerning genome evolution were hardly possible under these conditions. In contrast, the new ff-GISH method allowed a very clear and unambiguous labeling of the 2 parental genomes in *M. nayaritense* (fig. 2G; online suppl. figs. 1, 2).

Formamide-Free GISH

The effective melting temperature of DNA ($\text{Eff } T_m$) is controlled by the sodium concentration and is close to 100°C in water. Consequently, the corresponding hybridization temperature would be at 60 – 70°C which, over a prolonged period of time, causes deterioration of chromosome and nuclei morphology. Therefore, organic solvents, most commonly formamide, have been used in hybridization mixes to lower the effective DNA melting temperature by reducing the thermal stability of double-stranded polynucleotides. Hybridization in the presence of formamide can be performed at lower temperatures without compromising the stringency (1% of formamide reduces the melting temperature of nucleic acid duplexes by 0.72°C) [Meinkoth and Wahl, 1984]. Typically, hybridization mixes contain 50% formamide which allows hybridization at 37 – 42°C with relatively high stringency. Formamide, however, also slows down the rate of renaturation and thus prolongs the time of hybridization considerably [Berndt et al., 1996; Blake and Delcourt, 1996].

Formamide in high concentration with moderate heat denaturation has been established as the standard in situ hybridization protocol ('formamide protocol'). This treatment allows for high stringency, i.e. minor binding sites of DNA probes can be suppressed so that only specific binding site(s) remain labeled. However, formamide-free hybridization mixes have been successfully applied for hybridization of single-stranded oligonucleotide probes (so called Fast FISH) [Berndt et al., 1996; Durm et al., 1996]. It has been argued that omission of formamide enhanced the sensitivity of probe detection. Recently, a formamide-free ISH mix has been developed for gene aberration tests in diagnostics, but formamide has been substituted with other non-toxic organic solvents (e.g. ethylene carbonate) [Matthiesen and Hansen, 2012].

In the absence of formamide, hybridization time and temperature are the most sensitive parameters for the hybridization process. Previously it was demonstrated that

both these parameters quantitatively influence the hybridization behavior of α -satellite probes and how this effect can be used to discriminate major and minor binding sites [Durm et al., 1996]. However, additional factors, such as the degree of condensation and 'aging' of the chromosomal targets, the composition and the pH of the buffer, and the type of chemical modifications used to label the DNA probes were also suggested to play a role in optimizing the hybridization efficiency. Lower hybridization temperatures (40 vs. 70°C for human α -satellite DNA in the non-formamide mix) and prolonged hybridization times (2 h vs. 15 min) resulted in hybridization of nearly all α -satellite DNA loci (low stringency) [Durm et al., 1996].

In comparison to the standard formamide-GISH hybmix, our ff-GISH procedure resulted not only in a much better discrimination of parental genomes in GISH experiments but also in stronger signals in FISH experiments with non-genomic probes like satellite DNA *PaB6* or 35S and 5S rDNA probes (both in independent FISH experiments and after reprobing of the GISH preparations; data not shown). To test if this improvement is (partially) due to the changed denaturation conditions in the absence of formamide, we denatured the preparations in a Coplin jar in 70% formamide prior to hybridization with either standard or ff-GISH mix, leaving all other steps and conditions of ff-GISH unchanged. No obvious differences were noted after the denaturation in formamide, except for a slightly more distorted chromosome structure and morphology (fig. 2C, F, I). Thus, the better performance of the ff-GISH is the result of the hybridization itself rather than the denaturation. The use of the formamide-free hybridization mix for FISH should, however, be further optimized by increasing the temperature and decreasing the time of hybridization to obtain an optimal signal-to-noise ratio of the specific probe to be hybridized. The less target-specific the probe (e.g. specific retroelement family), the more stringent conditions should be applied.

The melting temperature of nucleic acid hybrids depends not only on formamide but also on the concentration of monovalent cations (especially Na^+) and the GC content of the hybridized region, among others [Meinkoth and Wahl, 1984; Blake and Delcourt, 1996]. The increased sensitivity of the formamide-free in situ hybridization presented here mostly relies on the higher $\text{Eff } T_m$ of DNA hybrids, but also on the very low final concentration of SSC ($0.02\times$). Low Na^+ concentration allows for faster renaturation of the DNA, while the absence of formamide increases the renaturation temperature. Calculations of

Eff T_m for both standard and new formamide-free hybridization mixes yielded very similar values: (a) 53.9 and 56.7°C, respectively, for 40% GC content of the hybridized regions, and (b) 62.1 and 64.9°C, respectively for 60% GC content. The homology percentages (stringency of hybridization) at a hybridization temperature of 37°C corresponded to 87.9 and 85.9% (for 40% GC) and 82 and 80% (for 60% GC) for standard and formamide-free hybridization mixes, respectively. Since we do not have detailed information about GC content of the analyzed genomes, it is difficult to address this aspect. However, all of these Eff T_m allow sufficient and efficient denaturation at a standard 72°C temperature.

Due to the low salt concentration and the absence of formamide, the hybridization efficiency is higher and the rate faster, which makes the whole procedure more efficient. Additionally, the low hybridization temperature and the prolonged hybridization time (overnight, mostly for convenience reasons in order not to extend the experiment over 10 h per working day) might allow maximal saturation of genomic hybridization. Different times of hybridization (10–24 h) have been tested, and they did not significantly change the efficiency of the GISH. It is possible that the time of hybridization could still be shortened.

In comparison, the GISH protocol applied to *Tragopogon* [Chester et al., 2012] and based on the formamide-free FISH protocol developed for maize [Kato et al., 2004; Birchler et al., 2008] also allowed labeling of the parental genomes both in *Prospero* and in *Melampodium* (online suppl. fig. 3). The differentiation of the 2 genomes was comparable to their differentiation after ff-GISH in *P. autumnale*, although slightly less pronounced. In *M. nayaritense*, however, although the parental genomes were differentially labeled, chromosome morphology after GISH was suboptimal due to significant DNA loss (as judged by very poor DAPI labeling). The 2 protocols, the newly presented ff-GISH protocol and protocol of Chester et al. [2012], differ in several steps, most importantly in the temperatures of denaturation (72 vs. 82°C, respectively), the hybridization (37 vs. 55°C, respectively) and stringent wash (42 vs. 55°C, respectively). The lower temperature of ff-GISH might be more appropriate and might prevent DNA loss from small chromosomes, as observed in *Melampodium* (chromosome size 0.85–2 µm in *M. nayaritense*) [Weiss-Schneeweiss et al., 2009], in comparison to medium-sized chromosomes of maize or *Tragopogon* (2.5–6 µm) [Chester et al., 2012] or larger chromosomes of *Prospero*. Additionally, the protocol optimized for *Tragopogon* recommends longer hybridization times

to increase probe signal intensity (36–48 h). The current ff-GISH protocol does not require prolonged hybridization times. Hybridization efficiency after 12 h does not differ significantly from hybridization after 24 h. Both protocols can thus be applied to discriminate closely related sub-genomes, although ff-GISH protocols would most likely perform better for taxa with small chromosomes and genomes.

Both of the labeled genomic parental DNAs used for ff-GISH bind to chromosomes of both parental genomes, albeit with different efficiency. Thus, it is difficult to discriminate the 2 genomes in images from single channels (online suppl. fig. 1). Similarly, the use of only one labeled genomic probe and the use of the other probe as unlabeled blocking DNA did not allow clear discrimination of parental genomes. Only in overlaid images the distinction of the 2 parental genomes is clearly visible (online suppl. fig. 1), similar in its principle to comparative genomic hybridization (CGH) [Pinkel and Albertson, 2005]. The ff-GISH signals in overlaid images are the result of differential saturation of 2 colors (2 probes labeled with different labels). A higher intensity of one of the probes' signals in a specific region of the given chromosome/whole chromosome indicates the prevalence of this parental genome-specific repeats and usually corresponds to less effective labeling of this region by the other parental genomic DNA.

This differential labeling of chromosomes with both colors indicates that the hybridization does not exclusively rely on preferential mapping of taxon-specific repetitive DNA sequences, similar to standard FISH/GISH. Additionally, particularly in the absence of low levels of unique sequence types, differential mapping of shared/similar sequence types provides a majority of differentiation. Shared types of repeats of both parental genomes will thus compete for the same/very similar targets, and the final differentiation will result from the efficiency of their competition, provided there is enough saturation and a minimal level of repetitive DNA fraction differentiation (<99% of repetitive fraction similarity in *Prospero*).

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