A new and “open access” chromosome approach to complex genomes: flow sorting of FISH labeled chromosome in suspension

Giorgi D., Farina A., Grosso V., Lucretti S.

in

Porceddu E. (ed.), Damania A.B. (ed.), Qualset C.O. (ed.).
Proceedings of the International Symposium on Genetics and breeding of durum wheat

Bari : CIHEAM
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 110

2014
pages 605-611

Session 7

Perspectives in structural and functional genomics

A new and “open access” chromosome approach to complex genomes: flow sorting of FISH labeled chromosome in suspension

Debora Giorgi[1], Anna Farina[1], Valentina Grosso[2], Sergio Lucretti[1]

[1] ENEA –CASACCIA Research Centre, Rome, Italy
[2] Present address: University of Naples Federico II, Portici, Naples, Italy

Abstract. A number of leading crop species are either polyploid and/or hold a large genome as a result of the accumulation of highly repetitive sequences. These features hamper the assembly of complex genomes in spite of the fast development of new sequencing technologies (Next Generation Sequencing) and the availability of powerful bio-informatic tools. The chromosome approach, by enabling genome dissection into single chromosomes or chromosome arms via flow sorting, can contribute to reduce genome complexity. In all plants, this approach is restricted to species, or special cytogenetic stocks, as in the case of wheat, containing chromosome types that differ in size from the standard complement. We have developed an overall robust and easy method called FISHIS (Fluorescence *In Situ* Hybridization in Suspension) which overcomes the constrain of chromosome size and DNA content differences, allowing chromosome flow sorting on the base of fluorescent hybridization labeling of chromosomes in suspension. FISHIS relies on readily available synthetic, fluorescently labeled repetitive sequences and on a kaline DNA denaturation. We show that the method can discriminate between and hence isolate the A genome from the B *genome* of durum wheat (*Triticum turgidum* subsp. d*urum*), a number of the chromosomes of the same species and o*f T. monococcum*, as well as the whole complement of the diploid wheat relative *Dasypyrum villosum* (L.) Candargy.

Keywords. Fluorescence In Situ Hybridization In Suspension-FISHIS – Flow molecular cytogenetic approach, chromosome flow sorting – Wheats – *Dasypyrum villosum – Haynaldia villosa. T. monococcum.*

Une nouvelle approche chromosomique aux génomes complexes en « libre accès » : tri par flux des chromosomes marqués FISH en suspension

Résumé. *Un certain nombre d'espèces cultivées parmi les plus importantes sont soit polyploïdes et/ou ont un grand génome en raison de l'accumulation de séquences hautement répétitives. Ces caractéristiques ralentissent l'assemblage de génomes complexes, en dépit du développement rapide des nouvelles technologies de séquençage (Next Generation Sequencing) et la disponibilité d'outils bio-informatiques puissants. L'approche chromosomique, en permettant la dissection du génome en simples chromosomes ou bras chromosomiques via le tri par flux, peut contribuer à réduire la complexité du génome. Dans toutes les plantes, cette approche est limitée à des espèces ou à des stocks cytogénétiques particuliers, comme dans le cas du blé, contenant des types de chromosomes qui diffèrent de par la taille du complément standard. Nous avons développé une méthode globale robuste et facile appelée FISHIS (Hybridation In Situ Fluorescente en Suspension) qui surmonte la contrainte de la taille des chromosomes et des différences de contenus d'ADN, permettant de trier par flux les chromosomes sur la base du marquage par hybridation fluorescente des chromosomes en suspension. La méthode FISHIS s'appuie sur des séquences répétitives synthétiques facilement disponibles, marquées par fluorescence et sur la dénaturation alcaline de l'ADN. Nous allons montrer que la méthode permet de discriminer entre, et donc, d'isoler le génome A du génome B du blé dur (Triticum turgidum subsp. durum), un nombre de chromosomes de la même espèce et de T. monococcum, ainsi que le complément entier du blé diploïde apparenté Dasypyrumrapport villosum (L.) Candargy.*

Mots-clés. *Hybridation In Situ Fluorescente en Suspension – FISHIS – Approche du flux cytogénétique moléculaire – Tri par flux des chromosomes – Blés – Dasypyrum villosum – Haynaldia villosa – T. monococcum.*

I – Introduction

In plant genomics new avenues have been disclosed by the rapid development of next generation sequencing (NGS) technologies and new bioinformatics tools (Metzker 2010, Trengen and Salzberg 2012, Edwards *et al* 2013). However, the assembly of huge genomes is still challenging because of the polyploidy origin and the high content in repetitive sequences of many plant species. The "chromosome approach" (Dolezel *et a*l. 2007) consisting in isolation of individual chromosome or chromosomes arms by Flow Cytometry (FC) analysis and sorting, offers a clue to reduce genome complexity. FC relies on the passage of chromosome suspension through the focus of intense light source. Optical parameters, such as light scattering and fluorescence, related to chromosomes morphology and DNA content respectively, are detected by the instrument and finally display as an histogram or as a two dimensional representation (dot plot) of the distribution of the different chromosomes. Critical for chromosome discrimination and flow sorting is the occurrence of a sufficient difference (at least 10%) in DNA content among individual chromosomes, a condition quite uncommon in plants. In humans, where differences exist in base-pair composition of individual chromosomes, two different fluorescent dyes, that binds preferably to: AT (Hoechst 33258) or GC (Chromomycin A3) rich regions have been used to discriminate similar sized chromosomes. In plants all attempts to base the chromosome sorting (following the animal model) on variation in their A-T and C-G content have failed because of the uniformity across plant genomes with respect to base pair composition, probably due to the high amount of repetitive sequences scattered all over the genomes. The fluorescent labeling in suspension of such abundant repetitive sequences could represent an alternative approach to the flow discrimination of similar sized chromosomes. We developed a reliable, fast and cost effective method for Fluorescence *In Situ* Hybridization. In Suspension (FISHIS) of plant chromosomes using Simple Sequence Repeats (SSR) as probe, which allows, for the first time in plants, chromosome sorting based both on total DNA content (size) and on a FISHIS specific chromosome labeling pattern. The discriminatory power of FISHIS combined with the high throughput of flow cytogenetic analysis and sorting gives rise to the new "flow molecular cytogenetics" approach which has allowed to purify, separately, the A and the B genomes of durum wheat and several of its individual chromosomes. Besides we have isolated chromosome $6A^m$ from *T. monococcum* and all seven chromosomes of the wild diploid species *Dasypyrum villosum* (L.) Candargy. The development of this method offers many analytical and preparative opportunities for the extension of current genomic technologies to large and complex genome species.

II – Material and Methods

1. Plant material

Grain of the diploids *Triticum monococccum* and *Dasypirum villosum* were provided by S. Pogna (CRA Agricultural Research Council, Italy,) and C. De Pace (University of Tuscia, Viterbo, Italy), respectively. Seeds of durum wheat (*Triticum durum*) cv Creso were obtained from P. Gentili (ENEA, Rome, Italy).

2. Cell cycle synchronization and preparation of chromosome suspensions

The procedure for cell cycle synchronization and for preparation of chromosome suspensions were according to methods of Dolezel *et al.* (1999) and Giorgi *et al.* (2013).

3. DNA probes

The following probes were synthesized and labeled by Eurofins MWG Operon (Ebersberg, Germany): $(GAA)_7$, $(AG)_{12}$, 5'-FITC-$(GAA)_7$-3'-FITC, 5'-Cy3-$(AG)_{12}$, 5'-Cy3-$(AAT)_7$ and 5'-Cy3-

$(AAC)_5$. The HPLC desalted oligos were resuspended at 1µg/µl in 10mM Tris, 1mM EDTA. The 18S-5.8S-26S rDNA clone pTa71 probe (Gerlach and Bedbrook, 1979) was labeled by nick translation using standard kits (Nick Translation Mix, Roche) following the manufacturer's instructions.

4. ISFISH

A. Alkaline denaturation of DNA

The extent of DNA denaturation was determined experimentally by the addition of NaOH to a 150µl aliquot of suspended chromosomes ($2x10^6$ chromosomes/ml LB01) labeled with 36µM acridine orange (AO), followed by flow cytometric analysis . AO stains single-stranded DNA red and double-stranded DNA green. Flow data were collected in the form of dot plots by plotting the ratio ssDNA/dsDNA fluorescence against dsDNA fluorescence for 10^4 chromosomes per sample (for details see Giorgi *et al.* 2013). The optimum denaturation treatment was set at pH13 for 20min, followed by a return to pH8.0 by the addition of 1M TrisHCl pH 7.4 and maintaining the suspension on ice for 1min.

B. Fluorescent labeling

The oligonucleotides probes were dissolved in 300mM sodium chloride, 0.3mM trisodium citrate (2XSSC) at a concentration of 160ng/ml and directly added to the chromosome suspension immediately after neutralization. The pTa71 probe after dilution in 2XSSC at a concentration of about 300-400ng/ml was hot denaturated for 5' at 95°C and then added to the chromosome suspension. In both cases the labeling reaction was carried out for 1h at room temperature, without any washing or centrifugation steps. After hybridization, the samples were diluted 1:1 with LB01 buffer counter-stained with 7µM DAPI and analyzed by flow cytometry (300µl final volume). For chromosome identification by fluorescence microscopy (Fig. 3), 4µl chromosome suspension was mounted in 30% v/v LB01, 70% v/v Vectashield (Vector Labs, Burlingame, CA) containing 7µM DAPI.

C. Flow cytometry and chromosome sorting

The Chromosome analyses and sorting were performed with a dual laser FACS Vantage SE flow cytometer (BD Bioscience, San Jose, CA) (for details see Giorgi *et al* 2013).

D. T Fluorescence microscopy

FISHIS labeled chromosomes and nuclei, before and after flow cytometric analysis and chromosome sorting, were visualized through a Nikon Eclipse TE2000-S epifluorescence microscope equipped with a Hg100 lamp and filter sets appropriate for FITC, DAPI and Cy3 fluorescence. The single images from each filter set were captured and digitized using a cooled NIKON DXM1200 color camera (Nikon Instruments Europe, B.V. Amstelveen, The Netherlands). Fluorescence images were superimposed after contrast and background optimization using ImageJ v1.45 (rsbweb.nih.gov/ij/index.html).

III – Results and Discussion

The first set of experiments was performed in durum wheat with the aim to define the best conditions for controlled DNA denaturation and labeling of chromosome in suspensions. The unwinding of the DNA double helix, induced by alkaline pH treatment of varied duration, was assessed via the flow cytometric analysis of the metachromatic shift, from green to red fluorescence, of a

chromosome suspensions stained by Acridine Orange (AO). The optimal combination proved to be a 20min treatment at pH13 (Giorgi *et al.* 2013).

Different concentrations of probe were evaluated first using a $(GAA)_7$ microsatellite labeled with either FITC or Cy3, which has been shown to be highly effective in generating FISH karyotypes of wheat. (Kubalakova *et a*l. 2005, Cuadrado *et al* 2008). An intense and well defined hybridization pattern was achieved in chromosome suspension (Figure 1) using 160ng/ml $(GAA)_7$-FITC probe and the distribution of $(GAA)_7$-FITC sites identified in the durum wheat complement (genomes A and B) was highly reproducible and consistent with that observed in standard FISH on slide (Pedersen and Langridge 1997, Kubaláková *et al*. 2005). Moreover multi-color labeling was achieved by using different probe combinations (Fig. 1).

Figure 1. Single and double target FISHIS of durum wheat cv Creso chromosome suspensions. a) Chromosome suspensions hybridized with (GAA)7-FITC; b) chromosomes and a nucleus after (GAA)7-FITC and (AAC)5-Cy3 dual labeling. Bar=10µm (from Giorgi *et al.* 2013).

FISHIS proved to be effective in chromosome labeling of several *Triticeae* species (table1). In particular chromosome suspensions of *T. durum, T. monococcum* and *D. villosum* have been used for the flow cytometric analysis and chromosome sorting after FISHIS labeling. In durum wheat the standard flow karyotype, based on DNA DAPI staining, show three main peaks only one containing a single type chromosome, the 3B (Kubalakova *et al*. 2005; Giorgi *et al.* 2013). After $(GAA)_7$–FITC labeling, up to ten chromosome clusters were resolved in the dot blot by the FC analysis (Fig. 2). The unequal distribution of the GAA SSR among the A and B genome of pasta wheat, with the A genome chromosomes less intensely labeled compare to those in the B genome, allowed for the ready separation of the two whole chromosome set (Fig. 2).

Moreover several chromosome type as 1A, 6A and 2B could be isolated at high level of purity, respectively >90%, >93%, > 93%. In the wild species *D. villosum* (2n=14 ; genome VV), a far relative of cultivated wheats, the $(GAA)_7$ distribution on the V genome has been recently investigated (Grosso *et al*. 2012) by standard FISH on slide, which revealed the GAA chromosome specific hybridization pattern and its high discriminatory power. Such observation suggest a possible use of $(GAA)_7$ for the flow molecular cytogenetic analysis of *D. villosum.* Its standard flow karyotype comprised four peaks, only one represented by a single chromosome type, the 6V (Grosso *et al.* 2012). After $(GAA)_7$–FITC labeling of *D. villosum* chromosome suspension a dot plot karyotype was generated in which all seven chromosomes could be individually identified and isolated at a level of purity > than 85% (Figure 3).

Other oligonucleotides, besides $(GAA)_7$ have been used in single or double target FISHIS experiment and proved to generate a labeling pattern able to discriminate and hence to allow flow sorting of different chromosomes. The (AG)12 microsatellite showed hybridization signals on chromosomes 3B, 4B, 5B and 6B of both pasta wheat allowing the flow sorting of chromosome 5B

(double strong band) and 3B to a purity level above 90 (Giorgi *et al.* 2013). In *T. monococcum* the $(ACC)_5$ *and the* $(GAA)_7$ oligonucleotides markedly label the chromosome 6V, (Megyeri *et al* 2012). Combining, by FISHIS, the two different oligonucleotides labeled with the same fluorochrome (Cy3) it was possible to increase the specific fluorescence emission of chromosome 6A and to flow sort it at high level of purity (Figure 4).

Figure 2. Biparametric dot plot analysis of durum wheat cv Creso chromosomes. The fluorescence intensity emissions from chromosomes stained with DAPI (DNA content) and FISHIS labeled with GAA)7-FITC are joint together into a bi-parametric dot plot where each dot represents a single particle (blue: DNA stained by DAPI; green: (GAA)7-FITC labeling). On the right is shown the different distribution of the GAA SSR among the A and B genomes of durum wheat. It allowed for the ready separation of the two whole chromosome set and of a number of single chromosome type (colored regions in figure) at high purity.

Different kind of repetitive sequences, other than oligonucleotides, were also used as FISHIS probes, i.e., the ribosomal DNA sequence from *T. aestivum* named pTa71. Such probe is known to label the 1B and the 6B chromosome of *T. aestivum* and allowed the flow sorting of both chromosomes together.

In plants, the reduction of the genome into its chromosomal components represents an effective means of acquiring the full genome sequence of polyploidy large genome such as that of bread wheat (Safàr *et al.* 2004). So far, the standard wheat DNA flow karyotype has delivered the purification of only a single entire chromosome, namely 3B, while the only way to isolate its whole chromosome complement is based on the exploitation of ditelosomic stocks in which one chromosome pair is replaced by its corresponding arms. A major drawback in this reliance on aneuploid stocks is that they have not been, and are unlikely ever to be developed for all but a small number of higher plant species. Moreover, such stocks are often developed using model varieties of low agronomic value. Here, we describe FISHIS a robust, rapid and low cost labeling method which combined with FC gives origin to the new" Flow Molecular Cytogenetic Approach" FMCA. Such approach allowed chromosome sorting to be independent from the use of aneuploid stocks, thereby potentially opening the access to the genome of all wild or cultivated species of interest. Besides, the sorted chromosomes can be used in a number of applications ranging from physical mapping to genomic studies using the NGS technologies, as recently reviewed by Dolezel *et al.* 2012.

Figure 3. Flow karyotyping (a) and chromosome sorting of the whole complement of *Dasypyrum villosum* after (GAA)7-FITC labeling (b)). On the right: (GAA)7 distribution on *Dasypirum villosum* chromosomes (from Giorgi *et al.* 2013).

Figure 4. *T. monococcum* 6A chromosome sorting. a) Double target FISH with $(GAA)_7$-FITC (green) and $(ACC)_5$ Cy3 (red) SSR on T. monococcum chromosome spread. Chromosome 6A: single and double target hybridization with $(GAA)_7$ and $ACC)_5$SSR labeled with different (b) and with the same fluorochrome (c). Flow sorted chromosome 6A after $(GAA)_7$Cy3 and $(ACC)_5$ Cy3 FISHIS labeling (d).

IV – Conclusion

In conclusion FISHIS, by the innovative FMCA approach, extend the possible applications of FC to potentially all species of interest once a high quality chromosome suspension and the proper probes are available.

References

Edwards D., Batley J., Snowdon R.J., 2013. Accessing complex crop genomes with next-generation sequencing. *Theor. Appl. Gen.,* 126, pp. 1-11.

Cuadrado A., Cardoso M., Jouve N., 2008. Physical organisation of simple sequence repeats (SSRs) in *Triticeae*: structural, functional and evolutionary implications. *Cytogenet. Genome Res., 120*, pp. 210–219.

Doležel J., Číhalíková J., Weiserova J., Lucretti S., 1999. Cell cycle synchronization in plant root meristems. *Methods in Cell Science*, 21, pp. 95–107.

Doležel J., Kubalàková M., Paux E., Bartoš J., Feuillet C., 2007. Chromosome based genomics in the cereals. *Chromosome Research,* 15, pp. 51–66.

Doležel J., Vràna J., Safaàř J., **Bartoš J., Kubalàková M, Simkova H., 2012**. Chromosomes in the flow to simplify genome analysis. *Funct. Integr. Genomics,* 12, pp. 397–416.

Giorgi D., Farina A., Grosso V., Gennaro A., Ceoloni C., Lucretti S., 2013. FISHIS Fluorescence *In Situ* Hybridization In Suspension and chromosome Flow Sorting Made Easy. *PLoS ONE,* 8: e57994.

Grosso V., Farina A., Gennaro A., Giorgi D., Lucretti S., 2012. Flow sorting and molecular cytogenetic identification of individual chromosomes of *Dasypyrum villosum* L. (*H. villosa*) by a Single DNA Probe. *PLoS ONE,* 7:e50151.

Gualberti G., Doležel J., Macas J., Lucretti S., 1996. Preparation of pea (*Pisum sativum* L) chromosome and nucleus suspensions from single root tips. *Theor. Appl. Genet.,* 92, pp. 744–751.

Kubaláková M., Kovářová .P, Suchánková P., Číhalíková J., Bartoš J., Lucretti S., Watanabe N., Kianian S., Doležel J., 2005. Chromosome sorting in tetraploid wheat and its potential for genome analysis. *Genetics,* 170, pp. 823–829.

Metzker M.L., 2010. Sequencing technologies: the next generation. *Nat. Rev. Genet.,* 11, pp. 31-46.

Megyeri M., Farkas A., Varga G., Kovàcs G., Molnàr L., Molnàr I., 2012. Karyotypic Analysis of *Triticum monococccum* using standard repetitive DNA probes and simple sequence repeats. *Acta Agronomica Hungarica,* 60(2), pp. 87–95.

Safár J., Bartos J., Janda J., Bellec A., Kubaláková M., Valárik M., Pateyron S., Weiserová J., Tusková R., Číhalíková J., Vrána J., Simková H., Faivre-Rampant P., Sourdille P., Caboche M., Bernard M., Dolezel J., Chalhoub B., 2004. Dissecting large and complex genomes: flow sorting and BAC cloning of individual chromosomes from bread wheat. *Plant J.,* 39, pp. 960–968.

Treangen T.J., Salzberg S.L., 2012. Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nat. Genetics,* 13, pp. 36-46.