A historical and modern perspective on plant cytogenetics

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Abstract

Plant cytogenetics has continued to flourish and make essential contributions to genomics projects by delineating marker order, defining contig gaps and revealing genome rearrangements. Here we review the field of plant cytogenetics from its conception through the eras of molecular biology and genomics. Significant advances in chromosome preparation, such as extended fiber-FISH, have greatly increased the axial resolution limits, while imaging and signal amplification technologies have improved our ability to detect small gene-sized probes. Combinations of traditional FISH technologies with chromatin immunocytochemistry serve to broaden the ability of plant cytogenetics to shed light on genome structure and organization. These advances are described, together with selected examples that illustrate the power of plant cytogenetics in guiding genome projects.

Keywords: *FISH; cytology; chromosome; BAC; microscopy*

CLASSICAL PLANT CYTOGENETICS

The field of plant cytogenetics was heavily influenced by Barbara McClintock's pioneering work on maize (*Zea mays*). Her method for unequivocal identification of individual chromosomes permitted major discoveries regarding the structure and dynamic behavior of the maize genome [1–6]. Using carmine-based chromatin staining procedures, McClintock showed that all of the individual chromosomes could be uniquely identified from a single meiotic nucleus with a combination of two metrics, the relative lengths and arm ratios of the chromosomes [2]. This approach proved useful for cytogenetic map development in other plant species, including rice (*Oryza sativa*) [7, 8], sorghum (*Sorghum propinquum*) [9], and tomato (*Lycopersicon esculentum*) [10, 11]. Plant species with similar-sized chromosomes, however, required the development of additional techniques for unmistakable cytogenetic resolution and karyotyping.

In 1968, Caspersson *et al*. used the fluorescent dye quinacrine to produce additional banding patterns, Q-bands, on plant chromosomes [12]. In 1972, Vosa and Marchi compared Giemsa C-banding to Q-banding on the chromosomes of bean (*Vicia faba*), keeled garlic (*Allium carinatum*) and maize [13]. Development of chromosome-banding techniques greatly improved the usefulness of somatic chromosomes, which are significantly easier to acquire than meiotic chromosomes. Giemsa staining techniques permitted the identification of individual rice prometaphase chromosomes [14], as well as karyotype development for diploid rye (*Secale cereale*) [15] and Emir barley (*Hordeum vulgare*) [16]. In an effort to improve cytological techniques for plants, Schweizer demonstrated that cold pretreatments enhanced chromosome visualization for most of the species studied, except for *Vicia faba* [17]. Even under optimal staining conditions, the ability to distinguish all chromosomes clearly can be hampered by the inherent morphological similarity of chromosomes in certain plant species [18–20]. Over many decades, variations of the carmine-based and banding techniques were adapted and optimized for cytogenetic characterization of different plant species

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[14–17, 20, 21]. These classical approaches have proven invaluable for chromosome characterization, but the development of *in situ* hybridization, which allows for direct visualization of specific DNA sequences on chromosomes, produced a quantum leap forward by combining cytology with molecular biology [22, 23].

DEVELOPMENT AND APPLICATION OF MOLECULAR CYTOLOGY IN PLANTS

The development of *in situ* hybridization (ISH) techniques opened up opportunities for cytogenetic analysis of essentially any species, regardless of its inherent chromosome morphology [24–27]. In plants, the use of radioactive tracer or modified nucleotides (attached to biotin, digoxigenin, or fluorescent moieties) to make ISH probes permits microscopic visualization and localization of complementary sequences in cells and nuclei and on individual chromosomes [27–32]. Direct and indirect fluorescence *in situ* hybridization (FISH) has been broadly applied over the last 25 years, as recently reviewed by Jiang and Gill [33]. Although FISH is commonly used to map unique or low-copy-number sequences, it is also used to localize repetitive sequence to produce chromosome recognition cocktails or explore genome relations in polyploid or closely related plant species [34–37]. The broad applications of FISH in structural, comparative and functional genomics place plant cytogenetics in a unique position to complement, accelerate, or guide plant-genome research [38–53].

SMALLER IS BETTER: STRATEGIES FOR IMPROVING THE DETECTION LIMIT WITH FISH

The power of cytogenetics is increasingly often focused on two related aspects of FISH, probe-size detection limit and axial-resolution limit. Here we use the phrase probe-size *detection limit* to mean the smallest FISH probe that can be clearly discerned and the phrase *resolution limit* to mean the smallest distance between two signals that can be resolved as separate and distinct in a microscopic image. Advances in microscopic sensitivity, signal increase and noise reduction have all contributed to improved detection limits, whereas advances in cytological resolution of closely linked loci (described below) are primarily derived from methods that lengthen the chromsome itself. Table 1 lists several key studies that highlight findings related to both of these issues.

Lowering the detection limit holds great promise for the common goal of being able to map cytogenetically any given single gene-sized DNA fragment such as a cDNA clone or an RFLP probe onto a chromosome. A key issue in detection limit centers on maximizing the signal-to-noise ratio, where the true FISH *signals* derive from the photons emitted by the hybridized probe molecules or their fluorescent ligands, and *noise* typically results from photons emitted by nonspecific or off-target fluorescence.

Table 1: Axial resolution and probe size detection limits in plant fluorescence *in situ* hybridization (FISH)

Cell type	Cell stage	Target chromosome		Axial resolution limit[c] (kb)	Probe size detection limit[d] (kb)	References[e]
		Chromatin[a]	Preparation[b]			
Somatic	Metaphase			2000–10 000	2.27–10	[33, 51, 52, 61]
		Euchromatin		4000–5000	>100	[61]
		Heterochromatin		5000–10 000	50–100	[52, 61]
			Superstretched	70	1000–2000	[65]
	Prometaphase			2000		[60]
	Interphase			100	10	[33, 55]
			Extended fiber	4.0	0.7	[60, 71]
Meiotic	Pachytene			100–40	3.1	[33, 49, 52]
		Euchromatin		120	50.0	[61]
		Heterochromatin		1200		[61]
			Superstretched	<50		[66]

[a]The type of chromatin is indicated for studies where it was specified.
[b]If other than conventional spread or squash technique.
[c]The smallest reported probe size resulting in detection of a FISH signal in a given study.
[d]The minimum reported distance between two FISH signals ordered and resolved along the chromosome axis.
[e]Primary or review paper describing the resolution- or detection-limit values.

Because of the sensitivity of high-resolution digital CCD cameras, detection limit is restricted not by probe size but rather by the signal that can be detected above the background noise. Routine detection of probes smaller than 1 kb remains difficult, although significant improvements in detection limits have been reported by many laboratories over the last 25 years [54–58].

Larger DNA segments, such as those from bacterial artificial chromosomes (BACs), are useful for producing stronger FISH signals, but these often contain repetitive sequences that may complicate the detection of specific target loci. Transgenomic FISH and pooled-BAC-PCR methods have been developed to improve specificity of detectable BAC FISH signals [42, 48, 50–52]. Figure 1 illustrates a modern application of FISH that builds on genomics resources to develop a cytogenetic map of maize. In this case, the genetic marker being FISH mapped, maize Core Bin Marker 1.05, CBM1.05 (csu3), is only 1.06 kb in size, near the practical detection limit for pachytene chromosomes. A maize marker-selected sorghum BAC clone is used as a surrogate probe to identify the cytological location of CBM1.05 (csu3) already linkage mapped to the short arm of chromosome 1. This example shows a set of image data with several types of FISH probes including a transgenomic BAC (arrow, Figure 1C and F), whole chromosome paint (Figure 1B), and repetitive centromeric DNA

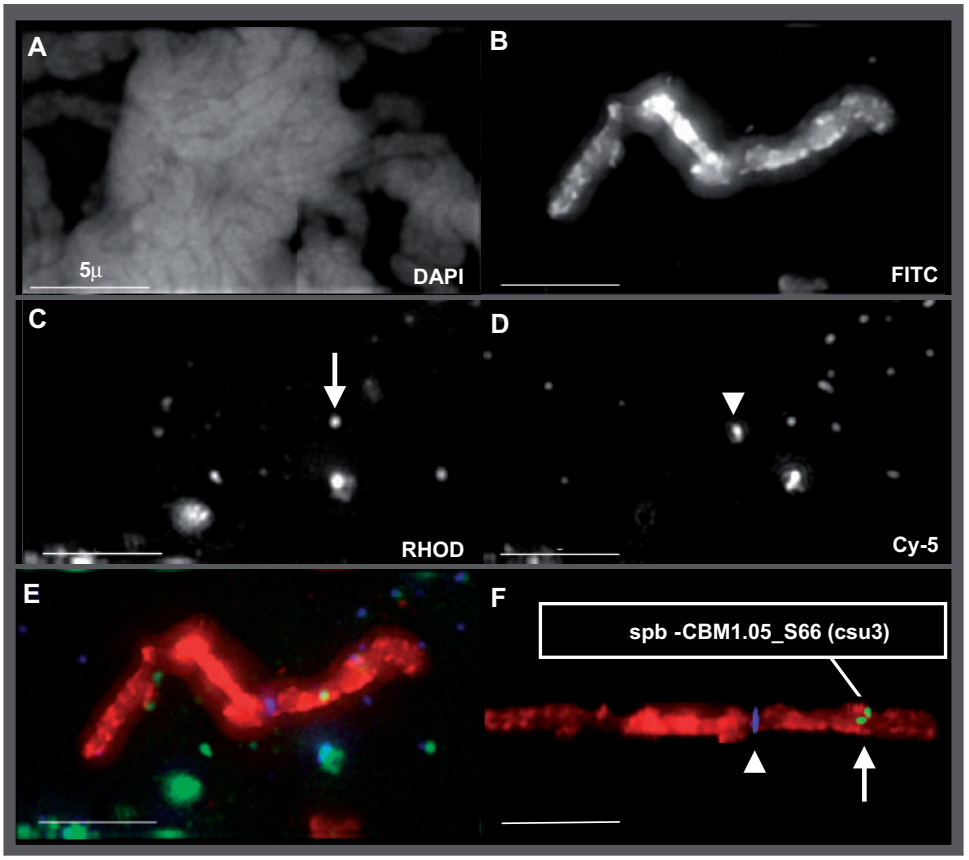

Figure 1: Fluorescence *in situ* hybridization (FISH) of a *Sorghum propinquum* BAC onto maize chromosome 1 in OMAd1.36, a maize-chromosome-addition line of oat. FISH mapping was carried out as previously described [48, 50]. (**A**) 4′,6-diamidino-2-phenylindole (DAPI) image of DAPI-stained pachytene chromosome spread from OMAd1.36. (**B**) Fluorescein isothiocyanate (FITC) image showing maize chromosome 1 hybridized with direct-labeled Alexa-488-dUTP total maize DNA. (**C**) Rhodamine (RHOD) image from direct-labeled BAC FISH signal (arrow) with the maize RFLP-selected *S. propinquum* BAC a0026E17. (**D**) Cy-5 image of centromere FISH signal (arrowhead) with direct-labeled CentC [103]. (**E**) Three-color overlay of the FITC (red), RHOD (green), and Cy-5 (blue) images. (**F**) Straightened projection of the maize chromosome with the three-color overlay scheme of panel E. The locations of the centromere (arrowhead) and CBM1.05 BAC FISH signals (arrow) are indicated, together with the resulting cytogenetic locus name (boxed). All scale bars are 5 μm.

(arrowhead, Figure 1D and F). The cytogenetic position of this maize marker is hereby determined to be at cytological position 1S.66 (Chromosome 1, Short arm, at 66% of the distance from centromere to telomere).

LOCATION, LOCATION, LOCATION: SPATIAL RESOLUTION IN PLANT CYTOGENETICS

Another common goal in plant cytogenetics is to resolve the location and arrangement of two or more DNA sequences in relation to each other along the axis of the target chromosome. Axial resolution typically refers to the ability to distinquish the relative positions of two loci as either *proximal* or *distal* to each other relative to the centromere. The axial resolution limit is usually reported in either base pairs or spatial units, such as the centiMcClintock, cMC [59]. These limits have been reported from various studies that used different probes and chromosome preparations, giving rise to apparent discrepancies, ranging from <0.7 kb in fiber-FISH to 10 000 kb in some metaphase(M)-FISH (Table 1 and references therein). These differences are attributed to variation in the axial compaction of DNA within the chromosomes and in the spacing of the probes used in a given study. Cleary the choice of chromosome type (e.g. somatic or meiotic) and how it is prepared (e.g. squashes or stretching) are primary determinants of resolution limit in a given experiment.

M-FISH based on plant meristem tissues, such as root tip, provides readily available material but can produce variable axial resolution depending on whether the probes are in euchromatic or heterochromatic regions (see Table 1 and references therein). Prometaphase chromosomes further improve axial resolving power [54, 60], whereas interphase-FISH (I-FISH) provides a reported resolution of ~50–100 kb [61, 62]. Despite having the poorest axial resolution, M-FISH remains a crucial method in plant cytogenetics for rapid assignment of cloned sequences to chromosomes and for ordering loci separated by at least several mega base pairs [33, 51, 52, 61].

Meiotic chromosome preparations, on the other hand, offer additional cytological landmarks and better axial resolution than do those from somatic cells [2, 10, 11]. Pachytene chromosomes are longer than their somatic counterparts by a factor of 6–25, depending on the species and method of chromosome preparation [11, 49, 63, 64]. Although meiotic chromosomes are not as readily available as somatic chromosomes, they are typically abundant and synchronized when isolated from pollen mother cells.

Methods specifically designed to unravel or stretch chromosomes before FISH have further increased axial resolution and allowed for finer-scale mapping of sequences than does conventional mitotic or meiotic FISH. Spreading interphase chromosomes for fiber-FISH, protease treatments of flow-sorted metaphase chromosomes, and superstretching of pachytene chromosomes are all methods that have enhanced our cytological view of chromosomal structures and sequence arrangements [65–67]. Fiber-FISH offers particularly high resolution (Table 1) and is used to characterize complex genomic arrangements in plant nuclei or plastids [67–70]. Given the average stretching degree of plant DNA fibers (3.12 kb/μm) [60, 67, 71] and the optical resolution limit of conventional epifluoresecence microscopy (200–300 nm), we can predict an axial resolution limit of ~600–700 bp.

CYTOGENETICS, IN THE POSTGENOMICS ERA

Plant cytogeneticists were among the earliest of genome researchers. They were visualizing genomes decades before the structure of DNA was discovered, 50 years before DNA cloning, and nearly a century before the first plant genome was sequenced [72]. The fundamental and classical cytological techniques remain excellent starting points for plant cytogenetic studies. Building on this foundation, FISH has extended our ability to identify specific chromosomes for almost any plant species with morphologically indistinguishable chromosomes [73, 74]. FISH permits rapid cytogenetic characterization and chromosome identification by means of a variety of probes such as those from repetitive DNAs, large-fragment clones, or closely related species. The recent development of cytogenetic maps of loblolly pine (*Pinus taeda*) [75] and Chinese bitter orange (*Poncirus trifoliata*) [76] nicely demonstrate this point. These maps were rapidly developed

from a combination of traditional chromosome preparations with FISH that used probes from BACs or conserved repetitive sequences from *Arabidopsis*.

Cytogenetic maps, which are based on the ultimate contigs, provide a unique conceptual framework for structural and functional genomics research. Molecular cytology offers an efficient means for sequence localization, validation of contig order and gap size, as well as characterization of complicated regions such as centromeres. For example, cytogenetic analysis of genetically mapped markers from the pericentromeric heterochomatin regions of maize chromosome 9 [52], tomato chromosomes 2 and 6 [77–79], and potato (*Solanum tuberosum*) chromosome 6 [79, 80] has helped resolved complex underlying genomic structures. Similarly, FISH has served to define heterochromatin–euchromatin boundaries in tomato [77, 78, 81], to localize centromeres relative to genetically mapped markers in both tomato [80] and maize [82], to produce high-resolution mapping within a contig in *Arabidopsis* [71], and to estimate the physical sizes of gaps between BACs in rice [60, 83, 84] and tomato [77, 78].

The use of cytogenetics to guide genome-sequencing efforts in tomato and potato also serves to illustrate the synergy between plant genomics and cytogenetics. Pachytene- and fiber-FISH were used to identify seed BACs for sequencing of tomato chromosomes 2 and 6 [77, 78]. FISH in tomato was used to investigate previously reported line-specific inversions and other discrepancies in genetically mapped marker arrangements [77, 78, 85]. More recently, similar FISH-guided strategies are being used to guide the BAC selection process as part of the potato genome-sequencing project [86].

FISH is also a powerful tool for comparative genomics, as beautifully demonstrated for members of the Brassicaceae [35, 87, 88], Solanaceae [79, 80] and Poaceae [42, 50, 89]. Fiber-FISH confirmed that *Arabidopsis thaliana* and *Brassica rapa* divergence was associated with chromosomal duplications [87]. In addition, comparative chromosome painting with pooled BAC probes was used to investigate ancestral relationships among species that diverged within the Brassicaceae [34, 35, 88, 90]. Collectively, these studies reveal the methods associated with plant cytogenetics to be uniquely informative and beneficial for genome analysis.

COMBINED TECHNIQUES AND THE FUTURE FOR PLANT CYTOGENETICS

The advances in microscopy, chromosome preparation techniques, and reagents for visualizing chromatin show great promise for plant cytogenetics, especially when used together. This concept is well illustrated by recent plant centromere studies in rice [91] and maize. For example, Zhong *et al*. [92] combined FISH, chromatin immunoprecipitation and immunocytochemistry to characterize the maize centromere-specific histone H3 variant, CENH3. They showed that CENH3 was associated with the kinetochore protein CENPC and characterized its propensity to colocalize with maize centromere-associated repetitive DNA elements CentC, CRM, and CentA. Jin *et al*. [68] used FISH and immunostaining to demonstrate that CentC and CRM sequences are interspersed at maize centromeres but that only a subset of these sequences were closely associated with CenH3. Zhang *et al*. [93] used extended chromatin fiber preparations to demonstrate that the CENH3-associated CentC sequences were relatively hypomethylated, whereas Koo and Jiang [66] developed and used a pachytene-chromosome superstretching technique to document the uneven distribution of this CentC hypomethylation at high resolution. Using combinations of methods such as molecular and chromatin cytology with new chromosome preparations and high-resolution imaging adds new insights and models for understanding chromosome organization at multiple scales.

In summary, we have described how plant cytogenetics plays a vital role in a wide range of modern research disciplines, from structural and functional genomics to comparative evolutionary biology. Emerging fields such as plant chromosome engineering also rely heavily on molecular cytological analysis [94–96]. New breakthroughs in imaging technologies, such as 3D structured illumination and stochastic optical reconstruction microscopy [97–102], offer even more hope for bridging the shrinking gap between the molecular and cytological views of the chromosomes and genomes of plants. Witnessing the integration of plant cytogenetics with rapidly advancing fields such as high-resolution imaging, epigenetics and genomics is exciting. Although grounded in techniques pioneered nearly a century ago, plant cytogenetics is still evolving, providing

crucial and integrative tools for genetic and genomic analysis of plant chromosomes and genomes.

Key Points

- Cytogenetics was built in part on historical studies of plant genome structure.
- Cytogentics provides a conceptual foundation for modern genomics.
- Molecular cytology uses clones sequences for fluorescence *in situ* hybridization to integrate molecular biology and genetics with cytogenetics.
- Plant cytogenetics is an indispensible tool for modern genome projects, providing rapid discovery or validation of physical maps and guiding efficient choice of bacterial artificial chromosomes for sequencing.

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