A maize root tip system to study DNA replication programmes in somatic and endocycling nuclei during plant development

Hank W. Bass¹, Emily E. Wear², Tae-Jin Lee², Gregg G. Hoffman¹, Hardeep K. Gumber¹, George C. Allen³, William F. Thompson² and Linda Hanley-Bowdoin⁴,*

¹ Department of Biological Science, Florida State University, Tallahassee, FL 32306-4295, USA
² Department of Plant and Microbial Biology, North Carolina State University, Raleigh, NC 27695, USA
³ Department of Horticultural Science, North Carolina State University, Raleigh, NC 27695, USA
⁴ Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC 27695, USA

* To whom correspondence should be addressed. E-mail: linda_hanley-bowdoin@ncsu.edu

Received 1 October 2013; Revised 26 November 2013; Accepted 5 December 2013

Abstract

The progress of nuclear DNA replication is complex in both time and space, and may reflect several levels of chromatin structure and 3-dimensional organization within the nucleus. To understand the relationship between DNA replication and developmental programmes, it is important to examine replication and nuclear substructure in different developmental contexts including natural cell-cycle progressions in situ. Plant meristems offer an ideal opportunity to analyse such processes in the context of normal growth of an organism. Our current understanding of large-scale chromosomal DNA replication has been limited by the lack of appropriate tools to visualize DNA replication with high resolution at defined points within S phase. In this perspective, we discuss a promising new system that can be used to visualize DNA replication in isolated maize (Zea mays L.) root tip nuclei after in planta pulse labelling with the thymidine analogue, 5-ethyl-2′-deoxyuridine (EdU). Mixed populations of EdU-labelled nuclei are then separated by flow cytometry into sequential stages of S phase and examined directly using 3-dimensional deconvolution microscopy to characterize spatial patterns of plant DNA replication. Combining spatiotemporal analyses with studies of replication and epigenetic inheritance at the molecular level enables an integrated experimental approach to problems of mitotic inheritance and cellular differentiation.

Key words: DNA replication, EdU, endocycle, flow cytometry, high-resolution microscopy, maize, mitotic cell cycle, root development, S phase.

Introduction

Eukaryotic DNA replication is distributed in both time and space, with different regions of the genome replicating at different times in S phase and at different locations within the nucleus (reviewed by Bryant and Aves, 2011; Gilbert et al., 2010). In multicellular organisms, gene-dense euchromatin regions replicate early, and repetitive heterochromatic regions replicate later within the S phase of the cell cycle (Rhind and Gilbert, 2013). It has been reported in mammals and Xenopus that early replication occurs in the interior of the nucleus while late replication mostly occurs at the periphery (Pope and Gilbert, 2013). There is also evidence in mammals that clusters of replicons, known as replication domains, are regulated as units that can be visualized in cells pulse-labelled with nucleotide analogues (Gilbert et al., 2010).

Chromatin structure and replication programmes change during development and cell differentiation. Several examples of developmental regulation of replication timing have been described in mammals (Gilbert et al., 2010; Pope and Gilbert, 2013).
is known in plants, but there is evidence that replication time and origin use are subject to hormonal control (Ishida et al., 2010; Bryant and Aves, 2011). A significant amount of information has been gathered about the proteins and epigenetic modifications associated with plant DNA replication (Shultz et al., 2007; Costas et al., 2011a,b), and a replication timing profile has been produced for Arabidopsis chromosome 4 for cultured cells (Lee et al., 2010). However, our understanding of the larger-scale dynamics of chromosomal DNA replication is still very limited for plants.

Although some aspects of the DNA replication process are broadly conserved across eukaryotes (Bryant and Aves, 2011; DePamphilis and Bell, 2011), studying replication in plants presents unique opportunities. Unlike animals, plant meristems are typically indeterminate and continue to give rise to new organs after the initial embryonic stage, allowing studies of genome duplication and maintenance in organized tissues rather than in cells artificially maintained in culture (Costas et al., 2011b). In addition, many plant tissues undergo developmentally programmed endocycles, in which S phase is uncoupled from mitosis resulting in multiple rounds of chromosomal DNA replication and increased ploidy. We have only begun to understand potential differences in chromatin structure, replication programmes, and epigenetic inheritance between mitotic and endocycle S phases in plants (Hayashi et al., 2013).

Maize (Zea mays) has several features, including well-characterized genetics and a fully sequenced genome, that make it an excellent plant system for studying DNA replication. The relatively large and distinct chromosomes of maize are well suited for studies of cytogenetics and subnuclear structure (Birchler and Bass 2009). In addition, given its prominence as a crop species worldwide, understanding how maize replicates its genome may have important agricultural implications. This is especially true for important seed tissues such as the endosperm of cereal grain species, which undergo programmed endoreduplication and produce large amounts of valuable products such as seed protein, starch, and oils (Sabelli and Larkins, 2009)

The maize B73 genome is similar in size (2.3 Gb) to the human (3.2 Gb) and mouse (2.5 Gb) genomes and is comprised of about 85% transposable elements (Lander et al., 2001; Waterston et al., 2002; Schnable et al., 2009). Interestingly, the maize genome is c.17-times larger than the genome of Arabidopsis thaliana (Poethig, 2001), which has been used as a model for plant DNA replication studies (Van’t Hof et al., 1978; Jacob et al., 2010; Lee et al., 2010; Costas et al., 2011a,b). In spite of the large difference in genome size, it is estimated that the maize and Arabidopsis genomes contain 32 500 and 25 000 genes, respectively—a difference of only about 30%.

Comparison of DNA replication in maize and Arabidopsis will lead to a better understanding of chromatin dynamics in higher plants. As a first step towards such an understanding, we discuss in this perspective paper some of the advantages, challenges and opportunities associated with investigating the spatiotemporal aspects of DNA replication in maize. In particular, we highlight the utility of our recently developed maize root tip system using a combination of fluorescent labelling, flow cytometry, and high-resolution 3D microscopy to characterize spatiotemporal replication patterns in nuclei from naturally cycling cells.

### Isolating and staging S-phase nuclei for analysis

#### EdU as a tool for plant research

In recent years, 5-ethyl-2′-deoxyuridine (EdU), a nucleoside analogue of thymidine, has been used increasingly to detect cells actively replicating their DNA. This technology takes advantage of a copper(I)-catalysed azide-alkyne cycloaddition (often referred to as ‘click chemistry’) first described by Rostovtsev et al. (2002) and Tornoe et al. (2002). Click chemistry can be used to detect EdU incorporated into newly replicated DNA by reaction of the terminal alkyne on EdU with an azide attached to a fluorescent dye such as Alexafluor 488 (Salic and Mitchison, 2008). Darzynkiewicz et al. (2011) reviewed the benefits of using EdU for detecting DNA synthesis compared to earlier techniques. In particular, the elimination of the acid or heat denaturation treatment needed for antibody detection of 5-bromo-2′-deoxyuridine, a commonly used thymidine analogue, greatly enhances maintenance of DNA and protein structural integrity (Darzynkiewicz et al., 2011).

Kotogágyi et al. (2010) were the first to look in depth at the feasibility of using EdU in plant suspension cultures and roots for flow cytometry and microscopy. Since then, a number of research groups have used EdU for plant cell-cycle studies, establishing its utility in a variety of plant tissues (Table 1). Most of these studies have addressed three broad areas: (1) the proliferation patterns and fraction of cells entering S phase in various plant tissues (Ayaydın et al., 2011; Kelliher and Walbot, 2011; Ilima et al., 2012; Kuznetsova and Sheval, 2013); (2) changes in DNA replication in response to various mutations, transgenes or chemical treatments (Vansraelen et al., 2009; Zhou et al., 2010; Schubert et al., 2011; Tschöp et al., 2011; Zhu et al., 2011; Xu et al., 2012, 2013; Bazin et al., 2013; Xiong et al., 2013); and (3) the characterization of replication patterns at a chromosome or a chromatin level (Heckmann et al., 2011; Greer et al., 2012; Xu et al., 2012; Hayashi et al., 2013; She et al., 2013). More recently, EdU labelling over discrete time intervals has been used in combination with confocal microscopy (Hayashi et al., 2013) or flow cytometry (E. Wear, L. Hanley-Bowdoin, and W. Thompson, unpublished data) to determine the average length of S phase in plant tissues.

EdU-labelling techniques have been combined with immunocytology and fluorescent in situ hybridization (FISH) to explore patterns of DNA replication, replication-associated epigenetic marks, and chromatin packaging in plant nuclei. Multiple groups have studied the progression of premeiotic replication in germ line cells using EdU in conjunction with DNA probes or antibodies that target centromeric or...
Table 1. Reports of EdU labelling of nascent DNA in plant systems

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Name</th>
<th>Tissue</th>
<th>Applications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>Thale cress</td>
<td>Cell culture</td>
<td>Flow cytometry, microscopy</td>
<td>Kotogány et al. (2010)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Thale cress</td>
<td>Inflorescence</td>
<td>Microscopy</td>
<td>Vanstraalen et al. (2009), Zhou et al. (2010), Zhu et al. (2011), Hayashi et al. (2013), Xiong et al. (2013), Xu et al. (2013)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Thale cress</td>
<td>Root</td>
<td>Microscopy</td>
<td>Vanstraalen et al. (2009), Zhou et al. (2010), Zhu et al. (2011), Hayashi et al. (2013), Xiong et al. (2013), Xu et al. (2013)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Thale cress</td>
<td>Root</td>
<td>Flow cytometry</td>
<td>Xu et al. (2012)</td>
</tr>
<tr>
<td>Cucumis sativus</td>
<td>Cucumber</td>
<td>Root</td>
<td>Flow cytometry</td>
<td>Hayashi et al. (2013)</td>
</tr>
<tr>
<td>Cucurbita pepo</td>
<td>Squash</td>
<td>Hypocotyl and cotyledon</td>
<td>Microscopy</td>
<td>Ilina et al. (2012)</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>Barley</td>
<td>Inflorescence</td>
<td>Microscopy</td>
<td>Higgins (2013)</td>
</tr>
<tr>
<td>Luzula elegans</td>
<td>Wood rush</td>
<td>Apical meristem</td>
<td>Microscopy</td>
<td>Heckmann et al. (2012)</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>Alfalfa</td>
<td>Cell culture</td>
<td>Flow cytometry, microscopy</td>
<td>Kotogány et al. (2010), Ayaydin et al. (2011)</td>
</tr>
<tr>
<td>Medicago truncatula</td>
<td>Barrel medic</td>
<td>Root</td>
<td>Flow cytometry</td>
<td>Bazin et al. (2013)</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>Tobacco</td>
<td>Cell culture</td>
<td>Microscopy</td>
<td>Kotogány et al. (2010), Tresch et al. (2011), Hayashi et al. (2013)</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Rice</td>
<td>Cell culture</td>
<td>Flow cytometry, Microscopy</td>
<td>Kotogány et al. (2010)</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Rice</td>
<td>Root</td>
<td>Microscopy</td>
<td>Kotogány et al. (2010), Hayashi et al. (2013)</td>
</tr>
<tr>
<td>Secale cereale</td>
<td>Rye</td>
<td>Root</td>
<td>Microscopy</td>
<td>Klemme et al. (2013)</td>
</tr>
<tr>
<td>Secale cereale × Triticum aestivum</td>
<td>Wheat–rye hybrid</td>
<td>Tiller</td>
<td>Microscopy</td>
<td>Greer et al. (2012)</td>
</tr>
<tr>
<td>Solanum lycopersicum</td>
<td>Tomato</td>
<td>Root</td>
<td>Microscopy</td>
<td>Kuznetsova and Sheval (2013)</td>
</tr>
<tr>
<td>Vicia faba</td>
<td>Fava bean</td>
<td>Root</td>
<td>Microscopy</td>
<td>Schuber et al. (2011)</td>
</tr>
<tr>
<td>Vitis berlandieri × V. rupestris</td>
<td>Grape hybrid</td>
<td>Cell culture</td>
<td>Microscopy</td>
<td>Kotogány et al. (2010)</td>
</tr>
<tr>
<td>Zea mays</td>
<td>Maize</td>
<td>Cell culture</td>
<td>Microscopy</td>
<td>Kotogány et al. (2010)</td>
</tr>
<tr>
<td>Zea mays</td>
<td>Maize</td>
<td>Immature tassel</td>
<td>Microscopy</td>
<td>Kelliher and Walbot (2010)</td>
</tr>
</tbody>
</table>

telomeric DNA, histone proteins, or developmentally related proteins (Greer et al., 2012; Higgins, 2013; She et al., 2013). In particular, Higgins presented detailed methods for EdU labelling and isolating barley inflorescences and subsequent immunolocalization and FISH preparations. Others have investigated the colocalization of EdU with DNA probes or histone marks and the overall chromosomal organization following mitotic replication on holocentric chromosomes (Heckmann et al., 2011) or supernumerary B chromosomes (Klemme et al., 2013). There has also been interest in identifying ‘early’ and ‘late’ replication patterns and associating them with euchromatic and heterochromatic regions of DNA (Heckmann et al., 2011; Greer et al., 2012; Xu et al., 2012; Hayashi et al., 2013). Most of the studies in Table 1 used microscopy to visually stage plant nuclei in S phase, which only distinguishes large differences in EdU labelling and does not provide measures of DNA content. As a consequence, visual staging of EdU-labelled nuclei cannot resolve them into different parts of S phase. The few studies in Table 1 that combined EdU labelling with flow cytometry were analytical in nature and did not use flow sorting to enrich for discrete S-phase populations. Hence, more precise methods for staging plant nuclei within S phase are needed for fine-scale observations and measurements of DNA replication patterns both spatially and in relation to chromatin features. To address this need, we developed a method whereby EdU-labelled nuclei are isolated using a flow cytometer to separate S-phase nuclei into sequential stages for 3D microscopic analysis. We combined this strategy with analyses of nuclei from cells at different positions in the developmental gradient of the growing maize root tip to establish a new experimental system for studying plant DNA replication. Approaches to visualize and analyse DNA replication patterns in time and space are emphasized here. We are currently using these approaches to compare DNA replication programmes in mitotic and endocycling plant cells.

**Flow sorting for temporal resolution within S phase**

Flow cytometers were originally developed for use with mammalian blood cells but evolved to include a wide range of applications in the plant sciences (reviewed by Galbraith, 2010). The introduction of the chopping procedure to release intact nuclei from plant tissue homogenates (Galbraith et al., 1983) made it possible to run a single particle suspension through a flow cytometer and sort nuclei with various fluorescent tags. This isolation method has been adapted to fixed tissues through the use of gentle blending (Lee et al., 2010), after which nuclei can be subjected to the click reaction to couple a fluorescent Alexa-488 azide to EdU incorporated into nascent DNA.

Many DNA replication studies have used a flow cytometer to isolate different fractions of S phase based on DNA content as measured by 4’,6-diamidino-2-phenylindole (DAPI)
or propidium iodide staining (Chen et al., 1995; Woodfine et al., 2004; Lee et al., 2010). While this is a useful technique, it is not suitable for some applications because it does not separate replicating from non-replicating cells or nuclei (Fig. 1B and D). By adding a second parameter to the flow sorting (e.g. EdU incorporation measured after coupling to an Alexa-488 azide), we can readily distinguish nuclei in S phase from other cell-cycle phases. EdU-labelled nuclei, even those in very early or very late S phase, are resolved by flow cytometry as an arc distinct from unlabelled G1 and G2 nuclei in a bivariate plot of DAPI versus Alexa-488 (Fig. 1A and C). Gating of the arc can be used to further separate S phase into early, middle, and late stages (Fig. 1A, rectangles labelled E, M, and L). This approach for staging nuclei allows us to start with an asynchronous population of nuclei and avoid artefacts sometimes associated with synchronization procedures such as sucrose starvation or incubation with nucleotide synthesis inhibitors (Cooper, 2003).

Some technical considerations must be addressed when combining flow sorting and high-resolution microscopy. First, the fixation strategy and buffers must be carefully selected to balance the need to ensure a single particle suspension for the flow cytometer with the need to preserve chromatin structure and nuclear architecture for downstream analysis. Second, depending on the experimental goals, the length of the EdU pulse can be important. For example, a short pulse will label DNA proximal to replication forks, while a long pulse will label more of the genome and include sequences

Fig. 1. Labelling actively replicating DNA in maize root tips. (A–D) Root tips from 3-d-old seedlings were pulse-labelled with EdU for 20 min, followed by fixation and nuclei isolation and the nuclei were analysed by flow cytometry using a BD Influx instrument equipped with 355 nm (UV) and 488 nm (blue) lasers. Bivariate plots of DNA content (based on DAPI fluorescence in the 460 ± 50 nm detection range) and EdU incorporation (based on Alexa fluor (AF) 488 fluorescence in the 530 ± 40 nm detection range) are shown in A and C. The corresponding univariate histograms of DNA content are shown in B and D, respectively, from 0–1 mm root segments (A, B) and 1–3 mm segments (C, D); the gates corresponding to early (E), middle (M), and late (L) subpopulations of S phase are indicated as rectangles for the mitotic cell cycle (A) and the endocycle (C). (E) Bivariate flow cytometry profiles of mitotic and endocycling nuclei from consecutive 1-mm root segments up to 5 mm labelled with EdU for 1 h: the identical black boxes in each profile indicate the gates used for analysis of the percentage of nuclei with 2C, 4C, and 8C DNA content shown in the root schematic below. (F) Merged confocal images of root tip cross-sections taken at the points indicated by the black arrowheads, showing DAPI-stained DNA (red), and EdU label in newly replicated DNA (green). Bar, 100 μm.
that are distal as well as proximal to active forks. In many cases, knowing the duration of S phase in the material under study will allow a more informed decision on pulse length.

Programmed endocycling in plants and the developmental biology of roots

Endocycling: a widespread phenomenon in plants

A shift from a mitotic cell cycle to endoreduplication or an endocyte, in which cells replicate their DNA without subsequent cell divisions, has been documented during development for roots, shoots, leaves, and endosperm of diverse plant species (Joubes and Chevalier, 2000; Lee et al., 2009; Hayashi et al., 2013). The formation of many specialized plant tissues and organs is known to require endocycling. Examples include trichomes, leaf epidermal cells, maize endosperm, and tomato fruit among many others (Joubes and Chevalier, 2000). Recently, Breuer et al. (2010) reviewed some of the developmental controls of the endocyte in Arabidopsis leaves and roots, emphasizing the importance of the endocyte for normal organ growth. They and others have also noted that the transition from the mitotic cell cycle to the endocyte is often coupled with a shift from cell proliferation to cell expansion and differentiation (Breuer et al., 2010; Hayashi et al., 2013).

Opportunity afforded by root tip organization

Developmentally distinct regions along the maize root have been identified morphologically (Baluska et al., 1990; Baluska and Mancuso, 2013). The tip contains dividing cells, followed by a region termed the ‘transition zone’ or ‘post-mitotic isodiametric zone’, located approximately 1–2.5 mm from the root apex. In this region, cell division mostly halts and cells begin to increase in length and width before reaching a zone of rapid elongation. Endopolyploidy has been observed for maize roots (Biradar et al., 1993; Ogawa et al., 2010). However, there are only a few reports that integrate ploidy information with plant root developmental programmes (Hayashi et al., 2013). Using flow cytometry, we found that the first 10 mm of the maize root tip contain 2C, 4C, and 8C cells, but very few cells of higher ploidy (data not shown). To increase our resolution, we dissected a series of 1-mm segments from the root apex up to 5 mm of EdU-labelled maize roots and examined the distribution of isolated nuclei in the mitotic cell cycle and the endocyte by 2-colour flow cytometry, as summarized in Fig. 1. The flow cytometry data were first filtered to remove cellular debris based on plots of side scatter (90° angle light scatter) and DAPI fluorescence. Identical gates were then set to analyse the percentage of 2C, 4C, and 8C nuclei in each root segment (Fig. 1E). This analysis revealed that the first 1 mm of the root consists primarily of cells in the mitotic cycle with 37% 2C, 52% 4C, and 4% 8C nuclei (n = 39 351). The endocyte is increasingly prominent beyond 1 mm, with the 2–3 mm segment containing 9% 2C, 44% 4C, and 38% 8C nuclei (n = 27 238). Both types of replication have essentially ceased by 4–5 mm from the tip. Strikingly, most maize root cells exit the cell cycle with either 4C (43%) or 8C (36%) DNA content, with only a small fraction (10%) having 2C content. We also observed the distribution of EdU-labelled cells across different cell types by imaging cross-sections at intervals along the root (Fig. 1F).

Our experiments established the feasibility of combining careful dissection and flow sorting to target either the mitotic cycle or the endocyte for a molecular analysis of DNA replication in an organized plant tissue. There are several advantages of studying DNA replication patterns using root tips instead of cell cultures. By focusing on zones with active division, we can obtain preparations highly enriched in nuclei undergoing replication. Fixed root tips also provide relatively clean nuclei preparations, ideal for flow sorting. In addition, the spatial separation of mitotic and endocycling cells, in combination with flow sorting, affords an opportunity to compare DNA replication programmes before and after a normal developmental transition between the two cell-cycle types.

Collection and analysis of 3D fluorescent images of nuclei

Flow sorting considerations for 3D imaging using multiple wavelengths

Formaldehyde is an excellent fixative for chromatin and has been widely used for decades to preserve nuclear architecture and chromatin structure for microscopy (Belmont et al., 1989; Swedlow et al., 1993). We have used deconvolution microscopy to obtain high-resolution fluorescent 3D images of fixed nuclei using optical sectioning. In the past, we often employed a 3D acrylamide FISH technique to preserve nuclei within cellular or tissue contexts (Bass et al., 1997, 2000a, b, 2003). However, we found that nuclei fixed in situ and then purified from the maize root tip system retained their generally spherical shape without acrylamide embedding, which considerably simplifies the mounting and imaging procedures. We also found that nuclei fixation with formaldehyde can be carried out in the presence of various buffers, including phosphate-buffered saline or Buffer A. Buffer A was specifically developed to preserve the native chromatin state for cytological analysis of nuclear architecture and chromatin structure (Sedat and Manuelidis, 1978; Belmont et al., 1989). The formaldehyde plus Buffer A chromatin fixation has been adapted for use in plants and is also compatible with additional staining techniques such as FISH or immunocytochemistry (Howe et al. 2013). Phosphate-buffered saline has the advantage of being more compatible with flow cytometry and, as such, is our buffer of choice for experiments that combine sorting and microscopy. In addition, nuclei separated by 2-colour flow cytometry are already fixed, labelled with Alexa-488, stained with DAPI and, thus, ready for immediate 2-colour imaging. Fixed and sorted nuclei are stable in various buffers, and can be stored for later use at 4°C for a few months in air-tight, light-free tubes (data not shown).
Other useful features of wide-field epifluorescence deconvolution microscopy with a sensitive CCD camera include the ability to collect two additional wavelengths using conventional red (Cy3, Rhodamine, Texas Red, ROX, Alexa-546) or far-red (Cy5, Alexa-694) fluorophores and obtain intensity data over a wide linear range (1–4096 counts/pixel before deconvolution) for every wavelength. These capacities enhance comparison of EdU-labelling patterns with those of other chromatin or nuclear feature staining methods such as in situ hybridization or immunocytochemistry.

Display of 3D image data on 2D media

Collection of multicolour data adds extra dimensions of information. For each wavelength, a series of optical sections are photographed using a CCD camera and saved at 3D digital data. The different wavelengths represent a fourth dimension. For 3D imaging of fixed samples, separate images are collected for each wavelength at each focal plane, before the stage is moved to the next optical section. The interleaved image data are sorted by wavelength and subjected to computational deconvolution to correct for chromatic aberrations (Chen et al., 1995). In this way, photon counts are conserved and redistributed within the 3D space of a cube of images, accomplishing a level of deblurring and brightening that distributes pixel signal intensities over a wide dynamic range. Linear scaling up to 32 000 counts can be achieved without the use of photomultiplier tubes or artificial gain. As a result, the deconvolved images contain considerable substructure (for example, within the heterochromatic knobs, as shown in Fig. 2). This substructure is visible even for regions of the 3D dataset that may have narrow portions (10%) of the intensity range in any given experiment.

In Fig. 2, a representative 2-colour (DAPI and Alexa-488) 3D image of a single nucleus from a somatic cell in late S phase illustrates several ways to visualize the information contained in a 3D dataset using 2D media, such as computer screens or the printed page. The 3D dataset is comprised of optical sections of pixels with X–Y dimensions of 0.07×0.07 μm, just below the resolution limit of this type of microscopy. The spacing between adjacent images is separated by a user-chosen Z-spacing at point of initial imaging, typically 0.2, 0.25, or 0.3 μm Z-step sizes. These dimensions provide oversampling in X, Y, and Z, and are optimized for the tradeoff between image file size and resolution. The X–Y magnification and the Z step size together define the dimensions of the individual volumetric pixels (voxels) of the dataset. These voxels have a single assigned spatial position but wavelength-specific intensity values. As a consequence, the data for different wavelengths can be viewed separately (Fig. 2B) or in pseudocolour combination. Images are typically adjusted for brightness and contrast using interactive linear scaling prior to producing a particular image for display.

A valuable method for 3D image display is to produce a through-focus projection of the entire data stack, as shown in Fig. 2B. Two methods of doing this are shown for comparison. The ‘average’ projection preserves the true average value of all the voxels at a given X–Y position, whereas the ‘maximum intensity’ method displays only the single maximum voxel value regardless of where it comes from in the Z-stack. The maximum intensity view appears sharper because it is a composite of the brightest features, but the average projection is mathematically more correct and, thus, more suitable for quantitative measurements. Another view that provides a useful overview of a multicolour 3D image involves the production and display of sequential projections (Fig. 2C). Here, the 3D cube of data is divided into equal-sized thick segments along the Z-axis and the resulting thick optical sections are displayed as a series of images representing adjacent through-focus projections. This allows for ready inspection of the entire 3D data in several parts, without having to view all 60 optical sections separately. Objects that appear adjacent in whole-nucleus projections (Fig. 2B, double arrows) can often be seen to be spatially separate when viewed in sequential projections (Fig. 2C, double arrows).

We found that both the DAPI and Alexa-488 (EdU) signals are very stable through additional staining techniques such as FISH (Fig. 2D), allowing for further colocalization studies within the nucleus. The images in Fig. 2 reveal variation in DNA replication patterns across the nucleus, including the previously reported tendency for heterochromatic knobs to replicate late in S phase (Pryor et al. 1980). The late replication of knobs is seen as overlapping signals for the Alexa-488 and the knob FISH probe (‘k’ in Fig. 2D indicates the locations of two late-replicating knobs). We also note that 3D imaging may reveal important but subtle differences in replication timing patterns, such as those associated with early versus middle S phase (H. Bass, G. Hoffman, T-J. Lee, E. Wear, S. Joseph, G. Allen, L. Hanley-Bowdoin, and W. Thompson, unpublished data).

Other useful ways to visualize 3D data include production of stereo pair projections (not shown) or movie files that move your view through or around the 3D dataset (Supplementary Table S1, available at JXB online). Exporting of 3D images to common movie file formats allows one to scroll through the optical sections (step-through movies) or ‘spin’ the 3D data using a series of projections viewed from different angles about the Y-axis (spinning projection movies). The 3D dataset shown for the FISH-stained nucleus (Fig. 2D) was used to produce step-through and spinning projections movies of single or combined wavelengths (Supplementary Table S1 available at JXB online). Interacting with 3D data in this way can increase comprehension of 3D multiwavelength data, as illustrated by the patchy labelling pattern in this late S-phase nucleus. This example also demonstrates that Click-It Alexa-488 labelling of EdU provides lasting signals marking the pulse-labelled sites of DNA replication. Combining 3D microscopy with flow-sorted temporal separation based on DNA content gives unprecedented access to spatiotemporal information on DNA replication timing.

Spatial and fluorescence signal measurements from image datasets

Another useful feature of 3D digital data collection is that the datasets can be used as raw material for experiments.
allowing for measurements and quantitative hypothesis testing that take advantage of the spatial resolution and dynamic range of signal intensity data. In the case of DNA replication time during S phase, it is often of interest to compare the replicated DNA captured with EdU pulse labelling to the total genomic DNA. Here, two types of
Fig. 2B. Here we illustrate the use of such techniques to quantify the differences between early and middle S phase. For instance, the early S-phase nucleus (Fig. 3A left) appears to have more separation between the DAPI and Alexa-488 signals than does the nucleus from middle S phase (Fig. 3A right). This difference was quantified using Pearson’s correlation coefficient (Fig. 3 legend) and the visual impression was verified numerically with a statistical R-value.

We also measured nuclear volumes and the non-uniform distribution of signals using image segmentation (Fig. 2B legend). The nuclear volume was segmented using computer-assisted or manual edge tracings and the resulting polygons—one per optical section—were connected to produce a solid surface object with spatial coordinates linked to that dataset. The solid object serves to delineate the inside and outside of the nucleus, providing information on nuclear volume, and the degree to which the DAPI and Alexa-488 signals are codistributed across the entire 3D dataset.

Together, the two approaches illustrated here—image projections for visual data inspection and collection of signal intensity and real-space measurements for quantitative analysis—are useful starting points for 3D cytology and particularly valuable for DNA replication studies. As large digital datasets become more common, sharing and reanalysing archived cytological data is expected to become more commonplace (Allan et al., 2012), and approaches similar to those illustrated here are expected to come into widespread use for 3D datasets.

**New insights and future prospects**

Using EdU and click chemistry to fluorescently label nascent DNA is an important technical advance in the general field of DNA replication. Although the EdU-labelling technology has been increasingly applied to plant systems (Table 1), its full potential is only now being realized with its adaptation to other technologies like flow cytometry. In this report, we discuss the experimental advantages for combined application of EdU and flow cytometry to label and fractionate replicating nuclei from maize root tips for DNA replication studies. Unlike cell-culture-based measurements of each optical section. Once completed, the series of all polygons (blue lines) are connected to form a closed surface that defines a solid object (green) that segments the voxel data into regions inside or outside of the object. The solid object is then used to calculate parameters such as real-space volume ($\mu$m$^3$), the centre of space (given as a single point with X-, Y-, and Z-values common to all wavelengths), or the centre of intensity (comparable to ‘centre of mass’, an intensity-weighted centre of mass with unique X-, Y-, and Z-values for each wavelength). The volumetric data provide quantitative values for observations that are otherwise estimates or subjective impressions.
systems typically used to study eukaryotic DNA replication, maize root tips provide a source of actively dividing nuclei undergoing a natural developmental programme in the context of an organized tissue. In particular, the maize root tip system can be used to examine DNA replication during the developmental transition from a mitotic cell cycle to an endocycle—a common transition during plant cell differentiation.

As described here, the maize root tip system can be combined readily with high-resolution microscopy to compare the spatial patterns of DNA replication during S phase in mitotic and endocycling cells. The maize root tip system can also be adapted to genomic protocols like DNA-IP, ChIP, and Hi-C to study replication programmes, chromatin landscapes, and long-distance interactions in EdU-labelled nuclei. These types of studies will help define the regions of the maize genome that replicate during early, middle, or late S phase and any associated chromatin features. Comparison of the temporal, spatial, and epigenetic properties of replicating DNA in maize root tips will provide insight into general characteristics of a plant nucleus in S phase and those that change when a cell transitions from a proliferating state to an endocycling state during differentiation.

Knowledge of the mechanisms that mediate and control DNA replication is essential for understanding plant growth and development. We now have the tools to explore these important processes in plants and to use this information to address important agronomic questions such as reducing phenotypic variation associated with passage through S phase or increasing grain yield by modulating DNA replication during endosperm development.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Table S1. Movies from a 4-wavelength 3D deconvolution image dataset of a nucleus in late S phase showing EdU-labelled DNA synthesis along with knob and centromere repeat FISH signals.

Acknowledgements

This work was supported by the National Science Foundation (grants IOS-1025830 to L.H.-B., W.F.T., and G.C.A., and IOS-1025954 to H.W.B.).

References


Bass HW, Bordoli SJ, Foss EM. 2003. The desynaptic (dy) and desynaptic1 (dsy1) mutations in maize (Zea mays L) cause distinct


