DNA synthesis and cell division in haploid maize shoot apical meristem cells during seed germination

Chuanmei Zhu, Yang Ju Im, Edward J. Cargill
Monsanto Company, St. Louis, Missouri 63167
Email: yang.ju.im@monsanto.com
Email: edward.j.cargill@monsanto.com

Abstract

DNA synthesis and cell division in the shoot apical meristem (SAM) tissues of germinating maize diploid seeds have been studied previously. However, little information is known about when and how the mitotic activity occurs in the SAM of haploid seeds during the germination process. In this paper, we studied the SAM cell division activity of dark growing maize haploid seeds. Using flow cytometry and histology experiments, we showed that the SAM of haploid maize seeds has the highest DNA replication activity at 48 h and reaches the maximal cell division rate (about 3%) at 72 h, mostly at the peripheral region of the SAM, shortly after radicle emergence. We conclude that cell division occurs after radicle protrusion is probably a general principle for both diploid and haploid seeds.

Introduction

During seed germination, the embryo inside of a seed grows into a seedling, with the shoot tissue derived from shoot apical meristem (SAM) cells and root tissue from root apical meristem (RAM) cells. Besides cell expansion, cell division is the major factor contributing to embryo growth and development. Therefore, it is fundamental to study when and how the meristem cells divide during seed germination.

Several papers have reported the cell division activity of SAM cells in diploid seeds during the seed germination process. In maize, it was shown that the SAM cells start to synthesize DNA 14 h after water imbibing and reach the highest activity at 40 h by measuring the incorporation of radioactively labeled thymidine into DNA (Baíza et al. 1989). In addition, it was shown that the first cell division in SAM occurs 28 h after water imbibing and the SAM reaches the maximal cell division rate (8% of cells dividing) shortly after the radicle protrustion at around 32 h (Baíza et al. 1989). In *Arabidopsis*, using flow cytometry (FCM) and cell cycle related gene expression studies, it was shown that DNA synthesis in germinating seeds occurs at the onset of root protrusion at 48 h and mitotic events are only observed after root protrusion (Barroco et al. 2005). However, in this paper, whole seeds were used in all the studies and thus the activity from SAM and RAM cannot be seperated. DNA synthesis and cell division activty in germinating seeds have also been studied in other diploid species, such as in wheat and barly (Gendreau et al. 2008; Benedetto et al. 1996). It seems that embryonic tissue division after radicle/root emergence is true among all the diploid plants (Barroco et al. 2005; Gendreau et al. 2008; Benedetto et al. 1996; Bewley et al. 2013). However, to our knowledge, there is no report on studying the cell division activity in haploid embryonic tissues during seed germination.

Haploid plant has been very useful in doubled haploid (DH) technology in plant breeding because it greatly shortens the breeding period for inbred line generation (Prasanna et al. 2012). A haploid plant grows smaller and is infertile by itself compared to a diploid plant (Prasanna et al. 2012). However, it is not known if the growth and development mechanisms between diploid and haploid plants are fundamentally different.

Here, we studied the mitotic activity in the embryotic SAM cells of dark growing haploid maize seeds. We found that DNA synthesis in embryotic tissues starts 24 h after water imbibing and reaches the highest activity at 48 h. In addition, cell division activity in the SAM is maximal at 72 h, shortly after radicle emergence, similar to diploid maize seeds.

Results and Discussions
Haploid seeds germinate at 48 h and grow most rapidly at 72 h.
We used one inbred line of haploid maize seeds for all the studies in this paper. Using a ragdoll germination method and dark growing conditions, we found that more than 80% of the haploid seeds germinate, defined by radicle protruding 5 mm or longer, between 39 h and 48 h after being put into the growth chamber (Figure 1A and 1B). By measuring the length of the coleoptile at different time points, we observed that the seedlings have an initial slow growth phase between 48-72 h, and grow fastest at 72 h with slowing growth after 96 h (Figure 1C).

48 h has the highest percentage of cells arrested in G2 phase.
To study the DNA synthesis activity in embryonic SAM cells of germinating haploid maize seeds, we employed a FCM technique, which has been widely used to study cell cycle from a large population of cells (Loureiro et al. 2008; Ochatt 2008). The caveat of this method is that the cells used in this experiment are not only from the SAM, but also from the surrounding leaf primordia, although we tried to remove the leaf primordial tissues as much as we can. Nevertheless, this high throughput method is still useful in telling us the status of nuclei in and near the SAM regions.

At 15 h, the majority of cells (>80%) are arrested in G1 phase, which is consistent with what has been reported from other literature (Barroco et al. 2005; Georgieva et al. 1993) as shown in Figure 2A, 2C, and Figure S1. From 15 h to 48 h, the percentage of cells in S and G2 phases increases (Figure 2C, Figure S1), suggesting that DNA replication occurs during this time period. After 48 h, the percentage of cells in G2 reaches a plateau and then decreases after 120 h (Figure 2C).

72 h has the highest mitotic activity.
To study when and how the SAM cells divide during the germination of haploid maize seeds, we performed histology experiments. We could only detect mitotic events in the SAM cells from 72 h seedlings (Figure 3A and 3B). The mitotic index is about 3% at 72 h (Figure 3A), indicating only a small number of cells in the SAM are actually dividing. Nuclei in different phases of mitosis were detected in the SAM of 72 h seedlings (Figure 3B). Notably, almost all the cell divisions observed occurred

at the base of the SAM (Figure 3B), *i.e.*, peripheral SAM. Cell division in the central SAM occurs very infrequently, which is consistent with what has been found in diploid seeds (Pautler et al. 2013). We did not detect any cell division before seed germination at 48 h or at 120 h, by which time seedling growth is almost stopped (Figure 1C).

Leaf primordia are differentiated from the peripheral SAM cells (Bowman and Eshed 2000). Similar to diploid maize seeds (McDaniel and Poethig 1988), there are 6 leaf primordia already existed in the mature haploid maize seeds. During seed germination process, there is a new leaf primordium emerging from the SAM (Figure 3C). We measured the length of this newly formed leaf primordium at different time points. We found there is not much growth of this leaf primordium before 72 h (Figure 3D), consistent with the finding that very few cell divisions occur during this period. In contrast, the primordium size increases dramatically after 72 h (Figure 3D), supporting that at 72 h the SAM has high cell division activity. In addition, despite some peripheral meristem cells differentiating to form leaf primordium cells, the size of the SAM is not changing (Figure 3E), further suggesting there is cell division at 72 h.

In summary, we studied the DNA synthesis and cell division activity in the SAM cells of haploid maize seeds during seed germination. We found that DNA synthesis occurs shortly after water imbibing, but division in SAM cells occurs only after root emergence. Different papers reported that DNA synthesis and cell division occur at slightly different times. This may be because different plant species, genotypes and/or germination methods were used in these studies. However, it seems that cell division happens after root emergence and is a general principle for many plant species. This seems to be independent of the ploidy level of the plants.

Methods and materials

Seed germination
Haploid maize seeds from a single inbred line were germinated as described in (Prasanna, 2012) with some modifications. Briefly, a row of seeds were placed on one layer of wet ragdoll germination paper. The germination paper was folded tightly into a bundle and tied with a rubber band below the seeds. The bundles of seeds were kept vertically in a plastic container with water covering the base of the paper. The plastic tube was placed in a Percival growth chamber, which maintained temperature at 25 °C under dark conditions. A seed was counted as germinated when its radicle was more than 5 mm in length. Seed germination rate was measured at different time points after seeds were put into the growth chamber. Coleoptile length of seedlings was measured using a ruler.

FCM experiment
Seeds/seedlings after germinating for different time points were harvested. Embryo axes were dissected out from ungerminated seeds and plummule (about 2 mm in length) were used for nuclei isolation. For germinated seedlings, about a 2 mm

region containing the SAM was cut out. Then at least the outermost three layers of leaves were dissected away in order to remove as much tissue as possible that is derived from pre-existing leaf primordia. The remaining inside tissue was used for isolating nuclei. A total of 20 samples were studied for each time point.

To extract nuclei, tissues were ground in 500 μl lysis buffer (15mM Tris, 2mM Na$_2$EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.5% Triton X-100, 0.11% β-mercaptoethanol and 54 units/ml RNaseA, PH=7.5) with 3/16'' steel beads in a 96 well plate at 1100 rpms for 30 seconds. Specifically, plummule tissues were ground at 1100 RPM for 2 minutes because 30 seconds is not sufficient to grind the tissue thoroughly. After grinding, samples were filtered through a filter plate and 10 μl 1mg/ml propidium iodide was added. Samples were then transferred to a 5ml culture tube for FCM analysis.

FCM analysis was performed using a BD FACSCalibur (BD Biosciences, San Jose, CA) and data was analyzed using Flowjo software. Fluorescence from 10,000 nuclei was measured. G1, S and G2 frequency was calculated using Watson model of the cell cycle analysis in Flowjo software. $S/N = f(G1)+f(S)+f(G2)/[1-(f(G1)+f(S)+f(G2))]$.

Histology experiment
Seeds/seedlings after being germinated for different time points were harvested. About 2 mm tissue above the mesocotyl containing the SAM was excised and fixed in 100% acetone for 30 minutes at room temperature. Acetone incubation would dehydrate samples. To rehydrate the tissue, the samples were incubated in 100%, 75%, 50%, 25%, 10% and 0% ethanol for 30 minutes, respectively. Tissue was mounted in cryo-gel and cut longitudinally into 50 μm sections. The sections were transferred onto a slide and washed with water and screened for those containing the SAM. The SAM-containing sections were incubated in 100 μg/ml promidium iodide solution for 30 minutes and then rinsed with water for 10 minutes. The sections were mounted in prolong anti-fading reagent (Invitrogen) and left flat at room temperature overnight. The slides were imaged on a Zeiss laser scanning confocal microscope with a 633 nm laser.

Acknowledgements

References

Baíza AM, Vázquez-Ramos M, Sánchez de Jiménez E (1989) DNA Synthesis and Cell Division in Embryonic Maize Tissues during Germination. Journal of Plant Physiology 135 (4):416-421. doi:http://dx.doi.org/10.1016/S0176-1617(89)80097-5

Barroco RM, Van Poucke K, Bergervoet JH, De Veylder L, Groot SP, Inze D, Engler G (2005) The role of the cell cycle machinery in resumption of postembryonic development. Plant physiology 137 (1):127-140. doi:10.1104/pp.104.049361

Benedetto JP, Ech-Chaoui R, Plissonneau J, Laquel P, Litvak S, Castroviejo M (1996) Changes of enzymes and factors involved in DNA synthesis during wheat embryo germination. Plant molecular biology 31 (6):1217-1225

Bewley JD, Bradford K, Hilhorst HM, Nonogaki H (2013) Germination. In: Seeds. Springer New York, pp 133-181. doi:10.1007/978-1-4614-4693-4_4

Bowman JL, Eshed Y (2000) Formation and maintenance of the shoot apical meristem. Trends in plant science 5 (3):110-115

Gendreau E, Romaniello S, Barad S, Leymarie J, Benech-Arnold R, Corbineau F (2008) Regulation of cell cycle activity in the embryo of barley seeds during germination as related to grain hydration. Journal of experimental botany 59 (2):203-212. doi:10.1093/jxb/erm296

Georgieva E, López-Rodas G, Hittmair A, Feichtinger H, Brosch G, Loidl P (1993) Maize embryo germination. Planta 192 (1):118-124. doi:10.1007/BF00198701

Loureiro J, Dolezel J, Greilhuber J, Santos C, Suda J (2008) Plant flow cytometry - Far beyond the stone age. Cytom Part A 73A (7):579-580. doi:Doi 10.1002/Cyto.A.20578

McDaniel CN, Poethig RS (1988) Cell-lineage patterns in the shoot apical meristem of the germinating maize embryo. Planta 175 (1):13-22. doi:10.1007/BF00402877

Ochatt SJ (2008) Flow cytometry in plant breeding. Cytom Part A 73A (7):581-598. doi:Doi 10.1002/Cyto.A.20562

Pautler M, Tanaka W, Hirano HY, Jackson D (2013) Grass meristems I: shoot apical meristem maintenance, axillary meristem determinacy and the floral transition. Plant & cell physiology 54 (3):302-312. doi:10.1093/pcp/pct025

Prasanna BM, Chaikam V, Mahuku G (2012) Doubled haploid techonolgy in maize breeding: theory and practice. CIMMYT, Mexico, D.F.
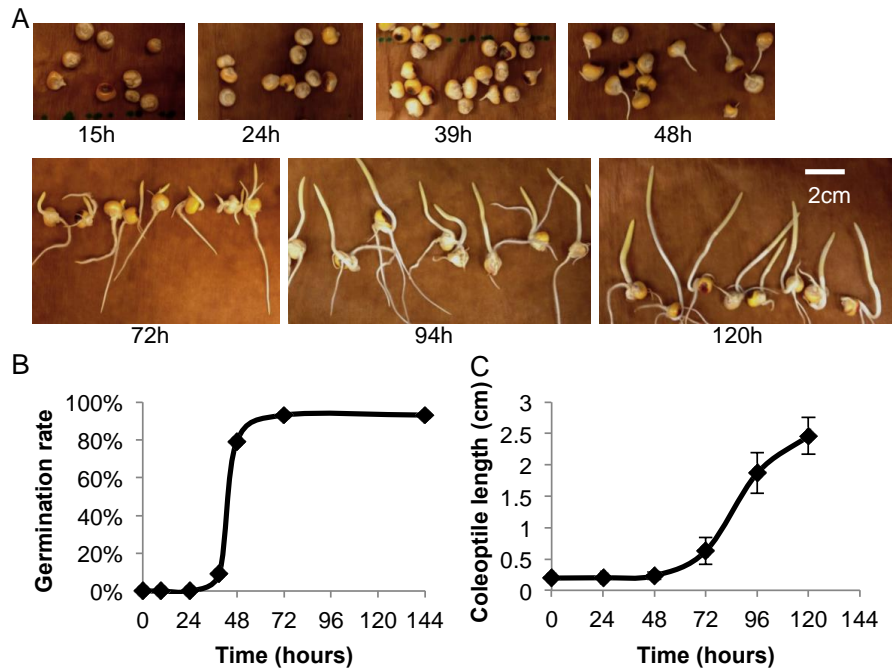
Figure 1: Maize haploid seed germination and growth
(A) Maize haploid seeds/ seedlings at different time points using ragdoll germination method. Scale bar=2cm.
(B) Maize haploid seed germination curve (n=30). Most seeds germinate after 48 h.
(C) Coleoptile length of maize haploid seeds/seedlings at different time points (n=20).
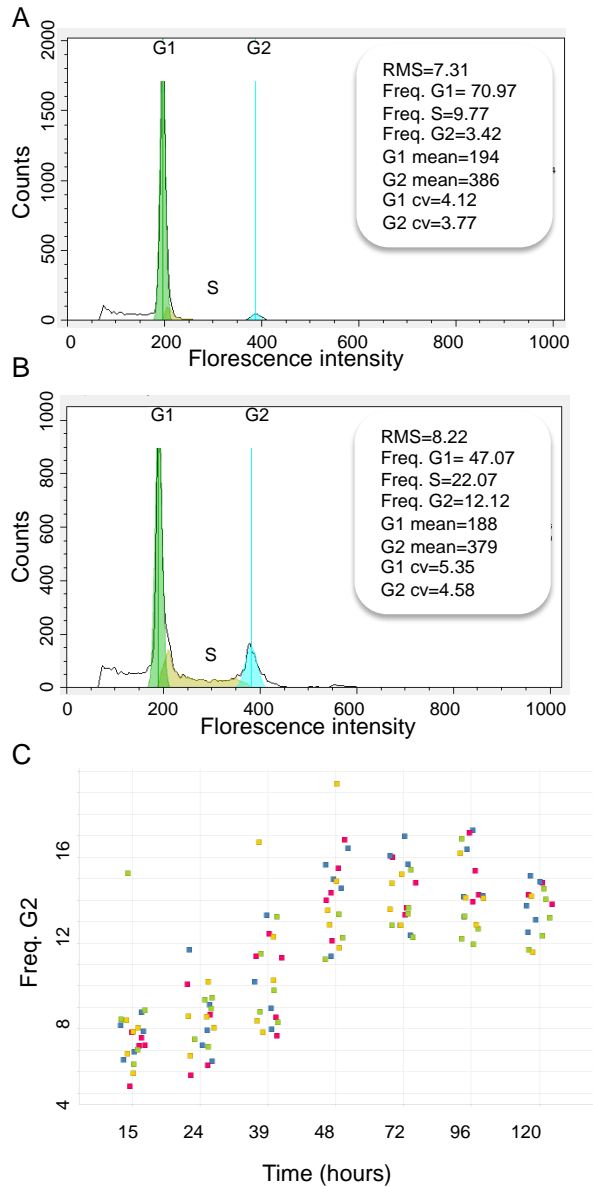
Figure 2: FCM analysis to study the DNA replication activity in the SAM
(A) and (B) Representative histograms of fluorescence intensity of propidium iodide-stained nuclei from samples that have been germinated for 15 and 120 h, respectively. The first peak shows G1 phase nuclei (before DNA replication). The second peak shows G2 phase nuclei (after DNA replication). In-between the two peaks shows S phase nuclei (undergoing DNA replication). The percentage of nuclei in different phases is shown as frequency. For each samples, 10,000 nuclei were counted (n=20).
(C) G2 frequency calculated from samples that have been germinated for different time points.
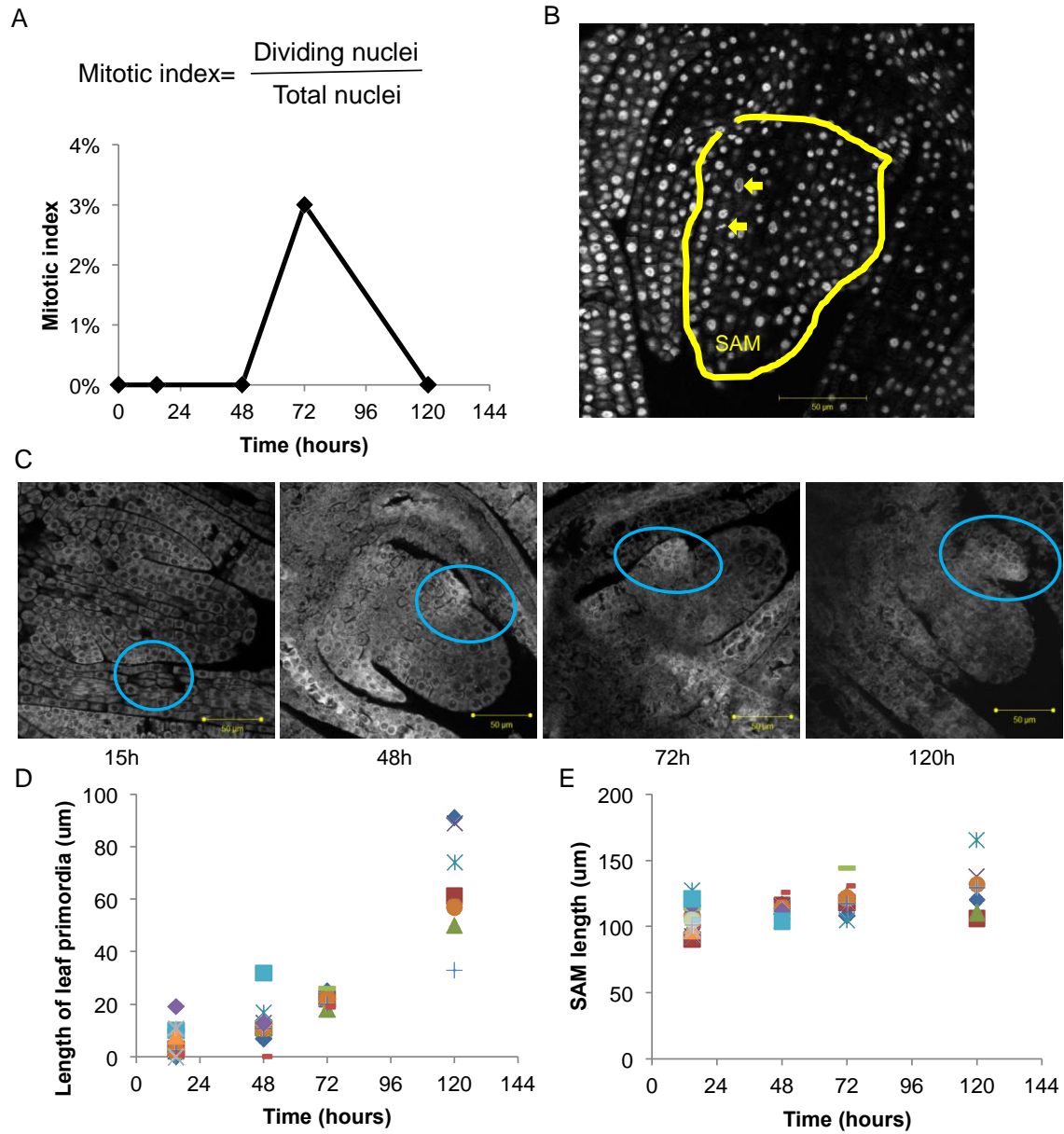
Figure 3: Histology analysis of cell division activity in the SAM

(A) Mitotic index of the SAM from seeds/seedlings that have been germinated for different time points (n=5). About 1000 cells were counted at each time point.

(B) Cell division events detected in SAM of 72 h old seedlings. Sample sections were stained with propidium iodide and imaged using confocal microscopy. The yellow region is the SAM. The yellow arrows show cells that are in prophase and metaphase, respectively.

(C) Representative pictures showing the structure of SAM from seeds/seedlings that have been germinated for different time points. The blue box shows the growth of 7th leaf primordium.

(D) The length of the 7th leaf primordium from seeds/seedlings that have been germinated for different time points (n=9).

(E) The length of the SAM from seeds/seedlings that have been germinated for different time points (n=9).
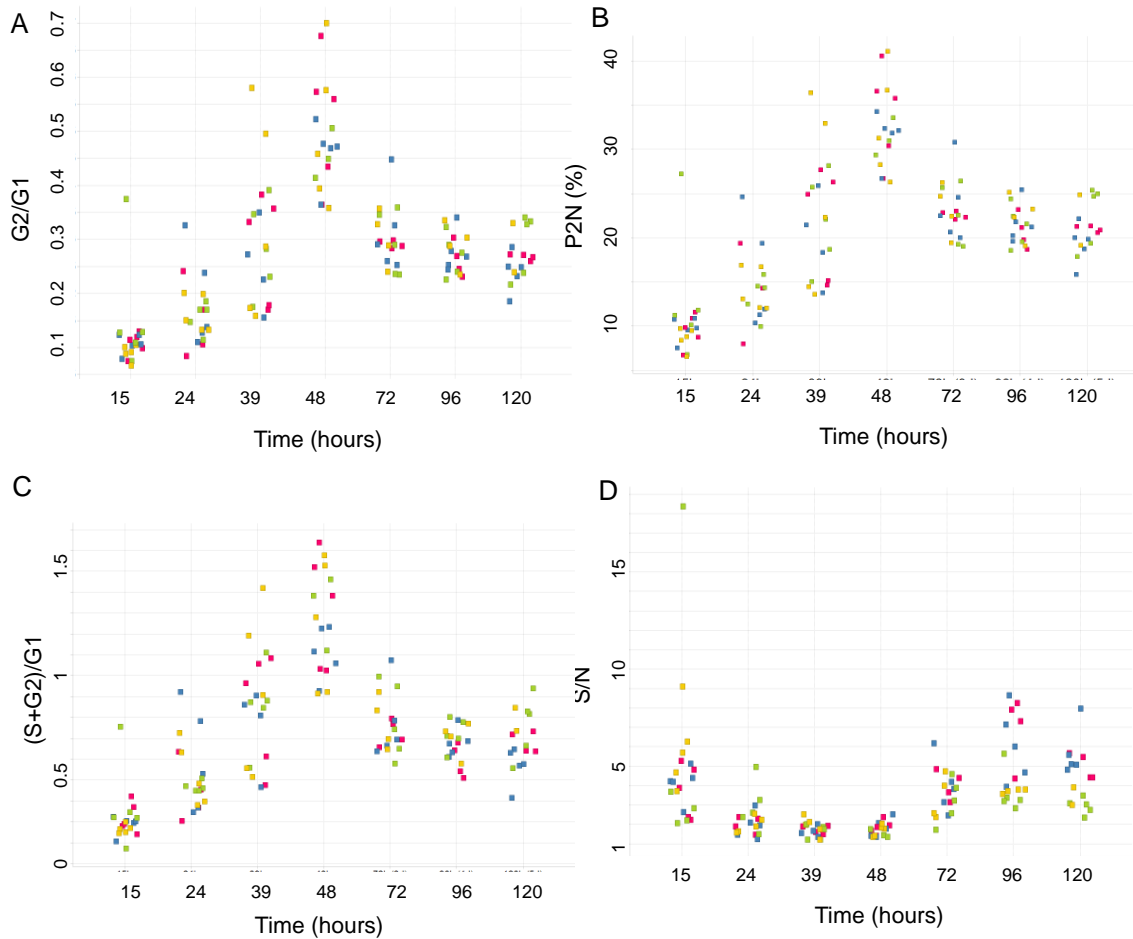
 Figure S1: FCM analysis to study DNA replication activity in SAM
DNA replication activity were plotted in other ways, G2/G1 (A), P2N (B) and (S+G2)/G1 (C), respectively. Signal to noise ratio (S/N) of the samples are also shown (D). Notably, the S/N value in 39 h and 48 h samples is lower than that in other samples, which may be because of nuclei damage after longer time of grinding in sample preparation. For this reason, the value of G2 frequency in 39 h and 48 h samples in Figure 2C may be underestimated.