

Short Communication

A Flow Cytometry Approach to Detect *In vivo* Chromatin Compaction from Plant Cells

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Abstract

It is now widely accepted that chromatin is a highly dynamic structure that participates in all DNA-related functions, including transcription, DNA replication, repair, and programmed cell death. Chromatin compaction influences plant regeneration and development. In plants, the most popular method of detecting chromatin compaction is immunofluorescence coupled with confocal microscopy. This method has great utility but still has limitations because the chromatin structure is subjected to change during nuclei isolation and treatment. To obtain *in vivo* status of the chromatin compaction of living cells, we explored a new method for detecting chromatin structure using flow cytometry to measure the chromatin compaction following quick protoplast isolation. This method can reflect the real condition of chromatin compaction *in vivo*. Using this method, we successfully and reproducibly separated various types of intact chromatin from soybean leaves at different developmental stages. The entire process is comprised of only three key steps: protoplast generation, Acridine Orange staining and flow cytometry. This approach can be completed within 6–8 h from protoplast isolation to flow cytometry measurement and shall be instrumental to plant epigenetic study.

INTRODUCTION

Mitotic chromatin compaction or 'condensation' has been well described for over 100 years. It is now widely accepted that chromatin is a highly dynamic structure that participates in all DNA-related functions, including transcription, DNA replication, repair, and mitosis; hence, chromatin compaction plays a critically important role in plant development. For example, it was reported that seed maturation in *Arabidopsis thaliana* is characterized by nuclear size reduction and increased chromatin condensation [1]. The heterochromatin condensation is observed during cell differentiation and maturation in human granulocytic lineage [2]. Chromatin modifications are also intensively studied in the context of imprinting and have recently received increasing attention in understanding the basis of pluripotency and cellular differentiation [3].

Evaluation of chromatin condensation is an important and stimulating topic. Cells, based on their conditions, can be divided into living or dead cells. For a long time chromosome banding pattern had been used to analyze the chromatin condensation [4]. This method depends much on chromosome separation by flake pressing and suffered from laborious work and a low success rate. To simplify the analysis of the chromatin compaction, other methods were also developed, which included electron microscopy and autoradiography approaches [5,6].

However, these methods required sample fixation which could cause chromatin structural collapse, leading to a low efficiency of chromatin compaction analysis and results were less reliable. Integrating microspectrophotometry was also devised to estimate chromatin condensation in mouse [7]. With rapid technical advances in super-resolution fluorescence imaging, the super-resolution fluorescence microscopy is now used as a tool to study the nanoscale organization of chromosomes [8]. Moreover, heterochromatin condensations can be determined by measuring the areas of heterochromatin regions using NIH image J. This method has enabled to analyze about 50 FGOs (fully grown oocytes) of control and PGC (primordial germ cells) null conditions [9].

All these above methods necessitate isolation of nuclei that can cause the collapse of chromatin structures leading to unreliable results.

How to measure the condensation rate of chromatin in living cells is an additional challenge in evaluating chromatin condensation. Biradar and Lane [1993] [10] developed a new assay approach to analyze chromatin condensations of isolated nuclei in maize by coupling flow cytometry with propidium iodide [PI] staining. They observed significant differences in fluorescence intensities among the diploid nuclei from the different plant organs. Mechanistically, use of fluorochromes with

different binding mechanisms enables to analyze the chromatin structure of the nuclei. The extent of chromatin condensation can thus be assessed by the degree of exclusion of the dye Acridine Orange, which binds to DNA [11,12]. This dye produces green fluorescence, when bound to double-stranded nucleic acids, but red fluorescence, when bound to single-stranded nucleic acid, under 480nm emission laser [11,12,13]. This method was successfully employed to detect the aberrancies or other defects in human sperm cells [14]. Very recently, sperm flow cytometry (SFC) has been used to evaluate the association of sperm chromatin condensation and ploidy with fertilization, embryo development and pregnancy [15].

Hyperchromicity of nuclear DNA as measured by maximal pixel fluorescence intensity allows one to identify cell types differing in degree of chromatin condensation such as mitotic or apoptotic cells. The laser-scanning cytometer (LSC) is a microscope-based cytofluorometer which has attributes of both flow and image cytometry. Laser-excited fluorescence emitted from fluorochromed individual cells on a microscope slide is measured at multiple wavelengths rapidly with high sensitivity and accuracy. Although the instrument has been available commercially for only several years, it has been already used in a various applications in many laboratories [16,17]. Flow cytometry using DNA-selective fluorochromes is now the prevailing method for the measurement of nuclear DNA content in plants.

A quantitative Förster resonance energy transfer (FRET)-based assay was developed recently to measure chromatin compaction [18]. This assay deploys multiphoton fluorescence lifetime imaging microscopy (FLIM) and is capable of measuring the compaction at the scale of nucleosomal arrays in live cells of human, though this method is technologically complex and costly to use at the moment.

Here we introduce a new approach for measuring chromatin condensation in living cells of plant by FACS™ analysis. This method is based on flow cytometry coupled with protoplast isolation while simple and quick. It doesn't cause typical problems including collapse of chromosomes caused by most of the methods described above. We have used this method to detect reproducibly the changing activities of nuclei during the cell growth and differentiation in *Arabidopsis* and soybean plant leaves. This approach should be a valuable tool to facilitate the study of epigenetic processes in soybean and many other plant species

RESULTS AND DISCUSSIONS

The isolation of soybean protoplast

Soybean protoplasts have been isolated from various tissues, for instance, leaf [19], hypocotyls [20], cotyledon [21], and callus [22]. The method described here is actually modified from the *Arabidopsis* protoplast isolation processes as described before [19,23].

Soybean leaves that represented five different developmental stages are chosen for our chromatin condensation analysis. Tissues of these stages include SAM (shoot apical meristem), young and folded, half-expanded, fully-expanded, and matured and old trifoliolate leaves, respectively (Figure 1). These different

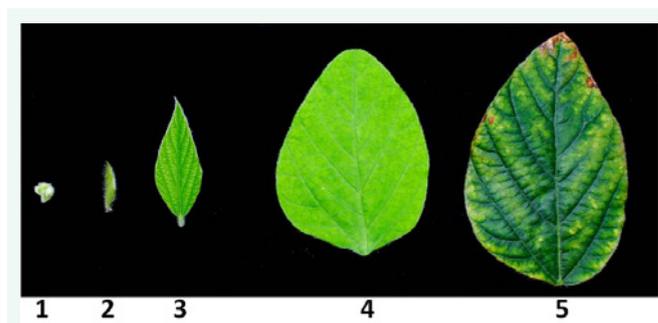


Figure 1 Leaf samples at different developmental stages.

1. SAM [shoot apical meristem]; 2. Folded trifoliolate; 3. Half-expanded trifoliolate; 4. Fully-expanded trifoliolate; 5. Matured and old [3 months] leaves.

stages may represent different chromatin epigenetic status which could influence cell pluripotency and regeneration. The leaf tissues were first sliced into 2mm strips and incubated in enzyme solution for 5 hours in dark. After 5 hours digestion, reaction solutions were filtered and most protoplasts were released from the leaf strips. Prolonged incubation of leaves (12–16 h) in the dark is not recommended in this protocol, because the longer culture time means more changes of cell growth condition and chromatin structure. The 5 hours digestion can produce $1\sim 2 \times 10^6$ protoplast/ml density, which is sufficient enough for flow cytometry analysis.

Flow cytometry and data analysis

Isolated soybean protoplasts are kept in Cell Permeabilizing Solution on ice and stained by 6ug/ml Acridine Orange solution following Golan's report [14]. A 488nm laser was used for double stranded DNA detection displaying green fluorescence while 488nm blue argon was used for single stranded DNA detection, showing red luminescence. In principle, a lower ratio of green vs. red fluorescence would represent less condensed chromatin because the more relaxed and active chromatin contain a higher percentage single-stranded DNA. We assume the RNA content in the same cells and treated the noise background as equal to 50,000 cells of each sample as detected by flow cytometry.

It should be noted that the chlorophylls in the protoplasts have a maximum emission at around 680nm. A significant emission at 640nm, which was used to detect acridine orange, can be observed. It is difficult to distinguish the Acridine Orange stained RNA from the chlorophyll if the protoplasts chlorophyll is excited by the 488nm laser and emits in the same channels. In order to address this issue, non-staining controls are prepared. Also, in flow cytometry, we avoid to run two fluorophores of the same excitation/ emission in the same channel. There are times when we can distinguish a high vs. low fluorescent signal in the same channel. This is the case where the chlorophyll is dimmer and the Acridine Orange staining DNA is a brighter in the same channel.

The forward scatter channel (FSC) intensity was used to distinguish between cellular debris and living cells; the side scatter channel (SSC) provided information about the granular content within a particle. A combination of the FSC/SSC plot was used to differentiate different cell types, and the granulocytes

with high identity were gated as region 1 (R1). The Region simply refers to an area drawn on a plot displaying flow cytometry data (Figure 2).

Two-parameter histogram, log height of red fluorescence versus log height of green fluorescence, was used as a quantitative measure for the chromatin condensation. This procedure was conducted as follows: on a scatter gram of red versus green fluorescence, region 2 (R2) was designed by drawing a polygon to include the majority protoplast from R1, excluding those with very low fluorescence (cell debris and also excluding cells containing abnormally high red:green fluorescence ratios (satellite populations) (Figure 3). The Green-Log Height/Red-Log Height of total granulocytes served as a measure of the degree of chromatin condensation. The high Green-Log Height/Red-Log Height ratio indicated condensed chromatin structures with higher percent of double-stranded nucleic acids, whereas low Green-Log Height/Red-Log Height ratio suggested a loose chromatin structure and even chromatin degradation.

The chromatin condensation of soybean leaf samples from five different development stages, those are from shoot apical meristem (SAM), folded trifoliolate, half-expanded trifoliolate, fully-expanded trifoliolate to matured and old (3 months) leaves were tested by this method.

Non staining samples were used as technique control to offset the chlorophyll fluorescence background. Our results showed that the SAM had lowest Green-Log Height/Red-Log Height ratio, which indicated a lower chromatin compaction; from folded trifoliolate to fully-expanded trifoliolate, these 3 stages had almost the same degree of chromatin compaction; the 3 months old leaf has the highest Green-Log Height/Red-Log Height ratio, which indicated a higher chromatin compaction status (Figure 4). From SAM to old leaves, the increasing Green-Log Height/Red-Log Height ratio indicated a condensing chromatin process, which corresponds to the requirement of gene expression in different developmental stages. The data also agree very well with well-

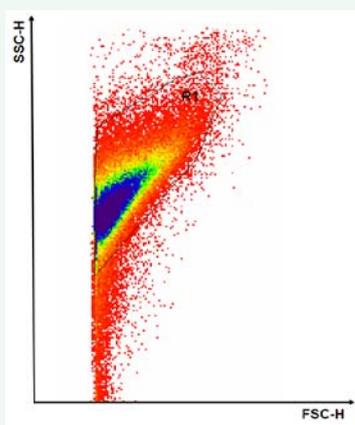


Figure 2 Physical characteristics analysis of protoplast using FSC/SSC. The samples were stained with Acridine Orange and analyzed by FACSTM. A combination of FSC height [FSC-H] and SSC height [SSC-H] was used to differentiate different cell types in a heterogeneous sample. The granulocytes with high identity were gated as region 1 [R1] in FSC/SSC plot;

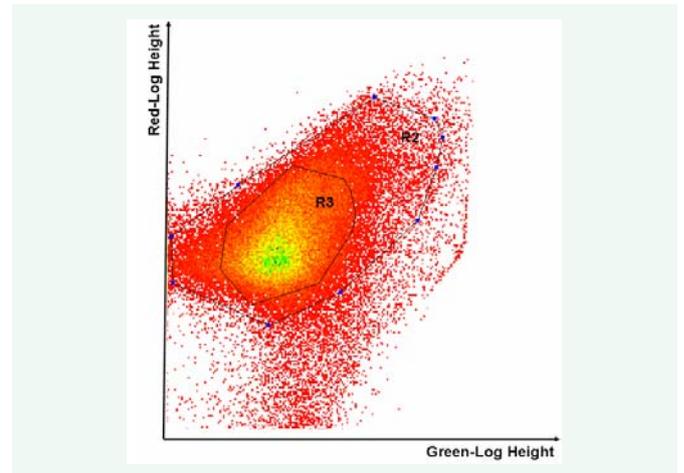


Figure 3 Two-dimensional scatter-grams plotting red versus green fluorescence was used as a quantitative measure for the chromatin condensation.

The granulocytes in R1 were further gated as region 2 [R2] and region 3 [R3], respectively. Region simply refers to an area drawn on a plot displaying flow cytometry data. The x and y axes display increasing log-value of green [Green-Log Height] and red fluorescence [Red-Log Height] intensities respectively. The Green-Log Height/Red-Log Height of total granulocytes served as a measure of the degree of chromatin condensation.

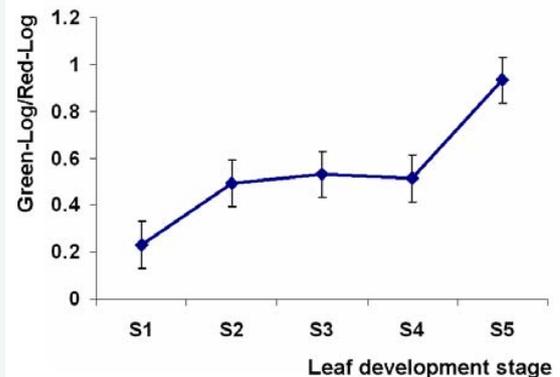


Figure 4 Chromatin condensation of leaf samples at different developmental stages illustrated by Green-Log/Red-Log.

The Green-Log/Red-Log was served as a measure of the degree of chromatin condensation. The younger tissue, shoot apical meristem tissue and folded trifoliolate tissue has a lower Green-Log/Red-Log ratio. As the leaf samples getting older, from S2 [young and folded] to S5 [3months old leaves], the Green-Log/Red-Log ratio going to highest, which suggested a condensing chromatin structure. Data are means \pm SD [standard error] [n = 3 biological repeats].

S1: SAM [shoot apical meristem]; S2: young and folded; S3: half-expanded; S4: fully-expanded; S5: 3 months old trifoliolate leaves.

known observations in plant tissue culture that younger leaves are generally more regenerable than older ones.

CONCLUSION

Flow cytometry coupled with a fast protoplast preparation can be used as a facile method to detect *in vivo* or near chromatin compaction from plant leaf cells. Such a method has not been

reported in plant previously. The whole process here we describe is quick, simple, and reliable and therefore should be instrumental for detecting chromatin compaction in epigenetic study of plant tissues such as leaf tissues where protoplast isolation can be rapidly achieved.

MATERIALS AND METHODS

Plant Growth

Soybean (*Glycine max* L.) plants were germinated and grown on either Metro-Mix 360 or Jiffy7 soil in 3-gallon pots in a greenhouse under controlled-environmental condition with 16/8h photoperiod at 25°C/ 18°C and light density of ~400 $\mu\text{E m}^{-2} \text{ s}^{-1}$. Plants were fertilized once with Osmocot 14-14-14 when the seeds were sown and watered as needed.

Protoplast Isolation

Healthy leaves from various development stages of soybean plants were harvested and cut into 1–2mm leaf strips from the middle part of a leaf and avoid vein. Immediately after cut, the leaf strips were transferred mixed with enzyme solution. The enzyme solution includes 20 mM MES, 1.5% (w/v) cellulase R10, 0.4% (w/v) macerozyme R10, 0.4 M mannitol, 20 mM KCl, and pH5.7. The solution was heated at 55 °C for 10 min to inactivate DNase and proteases and to enhance enzyme solubility. Then, the solution was cooled to room temperature [25 °C] and mixed with 10 mM CaCl₂ and 0.1% BSA. To achieve effective digestion, about 0.5g of leaf tissue in 15ml enzyme solution is recommended in a 50ml centrifuge tube and the enzyme solution containing leaf strips can be vacuum-infiltrated for 30 min in a desiccator in darkness. The digestion was continued in the dark for at least 5 h at room temperature. The enzyme solution should turn green after a gentle swirling motion, which indicates the release of protoplasts. The quality of protoplast preparation should be visually examined under a microscope. The enzyme/protoplast solution was diluted with an equal volume (15ml) of W5 solution before filtration to remove undigested leaf tissues and was filtered with 70 μm nylon mesh. The W5 solution includes 2 mM MES, 154 mM NaCl, 125 mM CaCl₂ and 5 mM KCl, pH5.7. The prepared protoplasts were re-suspended in 5ml MMG solution kept at room temperature for AO (Acridine Orange) staining. The MMG solution includes 4 mM MES, 0.4 M mannitol and 15 mM MgCl₂, pH5.7 whereas the AO staining solution includes 37mM citric acid, 126mM Na₂HPO₄, 150mM NaCl, 1mM Na₂EDTA, and pH5.7.

Acridine Orange Staining and Flow Cytometry

0.2-ml aliquot of the above protoplast suspension containing no more than 2 x 10⁵ cells was then transferred to a small glass or plastic tube (e.g., 2- or 5-ml volume) and added add 0.4 ml ice-cold cell permeabilizing solution. The cell permeabilization solution includes 0.1% (v/v) Triton X-100, 80mM HCl, 150mM NaCl, pH5.7. The solution was added and gently mixed. Immediately after the addition of AO staining solution the cell fluorescence was measured and recorded through the flow cytometer during the 2 to 10 min period at 488nm wave length.

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