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SHORT COMMUNICATION

Bromodeoxyuridine immunofluoresence and differential interference contrast imaging combination can precisely segregate adherent monolayer cells into specific cell-cycle phases

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Abstract: Most cellular-level cancer studies involve the identification of the cell cycle phases in which individual cells are progressing through. Traditional methods such as Fluorescent Activated Cell Sorting (FACS) require several treatments before harvesting the cells—procedures which alter cellular architecture. This study describes a novel method of the cell cycle analysis that preserves the cellular morphology and architecture with minimal *in situ* milieu perturbation. Primary rat skin fibroblasts were isolated and cultured at standard conditions. The cells were stained with anti-BrdU and examined with LSM 510 laser scanning microscopy. S-phase cells incorporated BrdU while M-phase appeared smaller and spherical. Damaged cells also tended to round-off in shape but, unlike M-phase cells, they did not bind anti-Phospho H3 antibody. G1 and G2 phases did not incorporate BrdU or Phospho H3. The two gap phases were differentiated on the basis of their sizes and subtleties in their shapes. The method is technically simple and less time-consuming while preserving the cellular *in situ* architecture. Due to its simplicity and accuracy the technique can be easily employed in resource-limited laboratories. Further studies are needed to verify the usefulness of the technique in clinical diagnostics such as cancer biomarkers.

Keywords: Cell cycle determination, monolayer cells, immunofluorescence

The hallmarks of cancer are the uncontrolled cell division process via various processes and the inherent immortality (Hannan & Weinberg 2000; 2011). A normally dividing cell progresses through four discrete phases—namely Gap 1 (G1), Synthetic phase (S), Gap 2 (G2) and Mitosis (M-phase). A fifth phase, G0 phase, is sometimes described referring to the terminally differentiated cells such as neurons and osteocytes.

Although the outcome of a cancerous process is most notable at the end of the M-phase of the cell cycle, most developmental derangements occur at the G1/S transition. Due to this fact most cellular-level cancer studies such as S-phase fraction (SPF) and DNA ploidy in tumour prognostication (O'Reilly *et al*., 1990; Chassevent *et al*., 2001) require the understanding of the cell cycle phases for specific individual cells. Traditional methods such as the Fluorescent Activated Cell Sorting (FACS) are the only reliable method through which the phase of the cell cycle can accurately be established. However these classical methods require many cells per sample and several cellular treatments during cell harvesting such as proteases which may invariably perturb the cellular morphology and physiological functions particularly in adherent monolayer cells (Schorl & Sedivy, 2007; Russa *et al*., 2009; Cappella *et al*., 2012). These methods, therefore, may not be suitably applied to the studies of monolayer cells that require the *in situ* milieu preservation.

Classically electron microscopic studies have been shown to segregate cells in their cell-cycle phases (Toth, 1981; Porter *et al*., 1973). However, they are time-consuming and unhandy and the distinguishing cell surface features can be inadequate (Lundgren & Roos, 1976). Overall, the cell treatment multiplicity involved in FACS and electron microscopic studies may hinder the understanding of subtle individual cell behaviour such as the cell cycle and perturb the optimal cellular function. Hence, asserting the phase of the cell cycle can be challenging particularly when studying adherent cells monolayer *in situ*.

The present paper outlines a simple novel method of which the phase of the cell cycle for individual cells can be easily established using a combination of nuclear imaging with Hoechst

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33342 and anti-Bromodeoxyuridine (BrdU) antibody staining of primary cell cultures with minimal perturbation hence modelling the *in vivo* cellular milieu. Co-staining with anti-Phospho Histone H3 (Ser 28) antibody for the detection of M-phase were further applied to improve the outcomes. The method is technically simple and less time-consuming while preserving the cellular *in situ* structure and function.

Skin fibroblasts were isolated from 6-10 weeks old Wistar rats (Wistar Institute**,** Pennsylvania, USA) using standard procedures in line with the Iwate Medical University Ethics in Using Animals in Research as detailed previously (Russa, 2011). Briefly the animals were euthanized using carbon dioxide (CO_2) gas. Quickly the abdominal fur was shaved off and the bare skin was disinfected with 70% ethyl alcohol. The abdominal skin was opened through a midline incision and the skin flaps reflected. Fat-free fragments ca. 5mm diameter were aseptically isolated from subcutaneous tissue and applied on 100-mm culture dishes. About 10 ml of Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 X1 (DMEM) (Gibco- Invitrogen, CA, USA) culture medium supplemented with 10% Foetal Calf Serum (FBS) was poured on each of the dishes to cover the culture tissue fragments and the dishes were maintained in a humidified incubator with 5% CO_2 flow at 37°C. After 48-72hr the cells had germinated and tissue fragments were removed from the culture dishes. Media were changed every 48 hours until cell growth reached confluence.

In order to obtain pure skin fibroblast culture cells were passaged 3-5 times before being harvested for use. Cell growth was assessed daily using a Phase contrast microscopy (Olympus, Japan). During the last circle of passaging, fibroblast were grown to 60-70% sub-confluence (24-48hrs) on 30mm (Ø) glass base dishes (Asahi Techno, Iwaki, Japan). Glass bottomed dishes (GBD) helped to image the adherent cells on a confocal microscope (LSM 510, Zeiss, Jena, Germany) *in situ* without harvesting the cells.

S phase cells were detected by incorporation BrdU into the cells using standard methods. Cells on glass-bottomed dishes (GBD) were treated with 10µM BrdU for 1 hour and then detected with anti-BrdU-Alexa Fluor-488 conjugated antibody as per manufacturer's instructions (Gibco-Invitrogen, CA, USA). BrdU is a thymidine analogue which is incorporated in the chromatin during DNA synthesis and hence labelling cells which are in S phase. Cells were counter-stained with Hoechst 33342 (Molecular Probes) for visualization of the nuclei—a procedure that stained all the stages of the cell cycle.

As a confirmatory measure for M phase cells in the specimen, the cells were further stained with Phospho histone 3 (Phospho H3) Serine 28 antibody (Sigma-Aldrich, Tokyo, Japan) and counter- stained with Propidium Iodide (PI). Phospho H3 is a confirmatory staining method for Mitotic Index (MF) determination—a clinicopathological procedures used in the prognostication of different tumours (Veras *et al.*, 2009).

To examine cellular morphology, cells on GBD were examined using Differential Interference Contrast (DIC) microscopic and fluorescent laser scanning techniques. Samples were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 10min at room temperature. Cells were then permeabilized with 1% Triton X in PBS for 5min. They were then washed with PBS and incubated with Alexa Flour 488 labelled anti-mouse IgG secondary antibody (Molecular Probes) at 1:200 dilution for 20min at room temperature. The samples were thereafter washed twice in PBS and counter-stained with 2 µg/ml Hoechst 33342 fluorescent dye in PBS for 5 min at room temperature and examined with a confocal laser scanning microscope (LSM 510). Hoechst 33342 and Alexa Fluor 488 were excited at 405 nm and 488 nm wavelength respectively (Figure 1).

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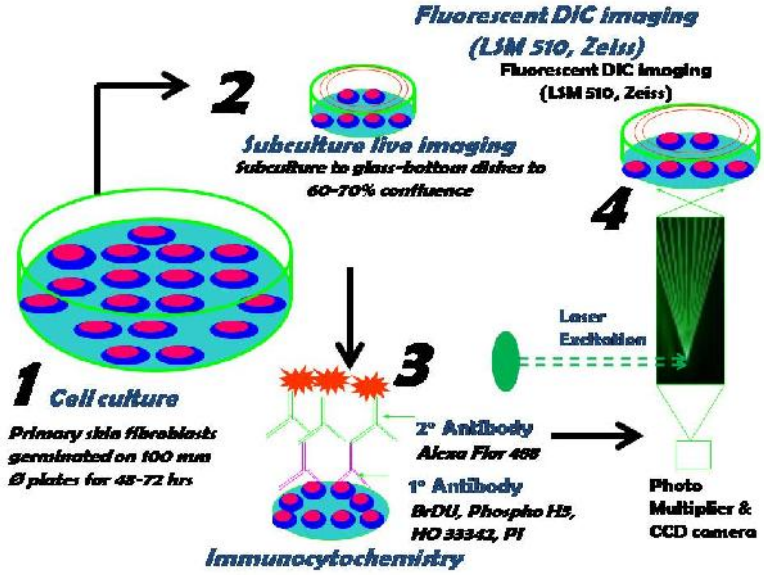

Figure 1: Schematic presentation of Methods summary.

The primary skin fibroblast had stallate morphology (Figure 2A) typical of primary mesenchymal cells. Passaging did not generally affect the morphology of the cells—entailing a minimal to no-effect of the culture conditions to the cellular functions and growth characteristics (Figures 2B and 2C). Cell population per area (density) tended to influence growth rate. Cells tended to grow in whorls probably due to contact stimulation. After several passaging, growth capabilities started to decline and cell shape and quality diminished. The cell growth rate was markedly reduced as majority of cultures failed to attain confluence and cellular organelles such us nucleolus, endoplasmic reticulum, which are normally visible in growing cells, were less pronounced (Figure 2D).

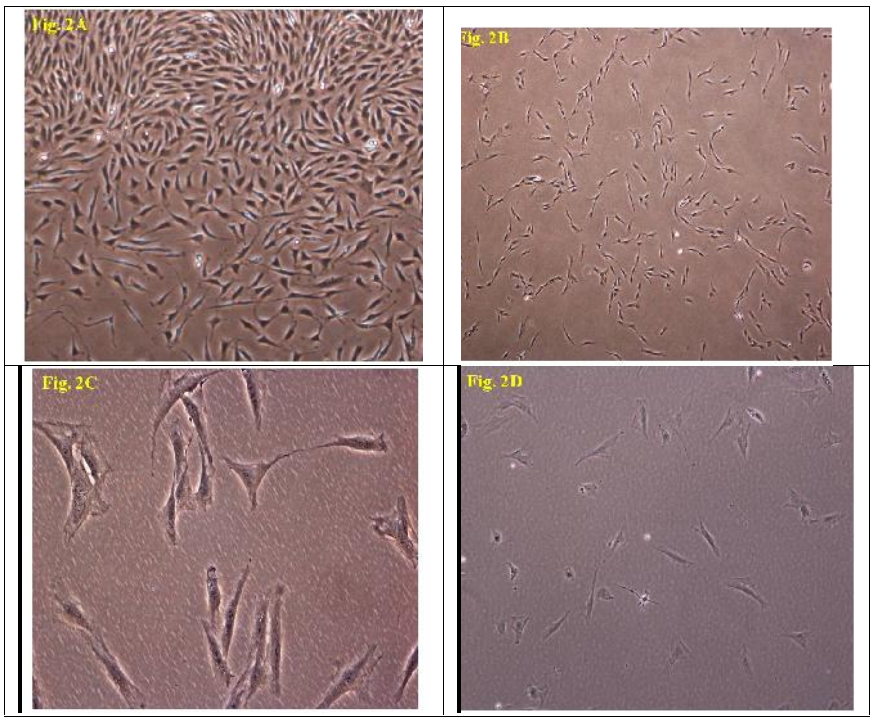

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Figure 2: Skin fibroblast isolation and culture under phase-contrast microscopic observation. A, Confluent culture before passaging; B, Sub-confluent cells after passaging (low magnification); C, Sub confluent cells after passaging (high magnification); D, Poor growth following repeated passaging

Gap Phase 1 (G1) cells were identified as ones with a relatively small and round nucleus on basic nuclear staining with Hoechst 33342 (HO342) (Figure 3A). Unlike the cells in the Synthesis Phase (S phase), the cells did not incorporate BrdU (Figure 3B). The G1 Phase cells differed from Gap Phase 2 (G2)—both being in a rest phase of the nuclear activity—by their perfectly round nuclei and a flat surface on the Differential Interference Contrast (DIC) image (Figure 3C). Further, G1 cells typically showed diffuse and homogeneous nuclear staining with HO342—unlike G2 which depicted rather particulate chromatin on HO342 staining (Figure 3A). The G1 phase did not stain with Phospho Histone 3 Serine 28 antibody (Figure 4D).

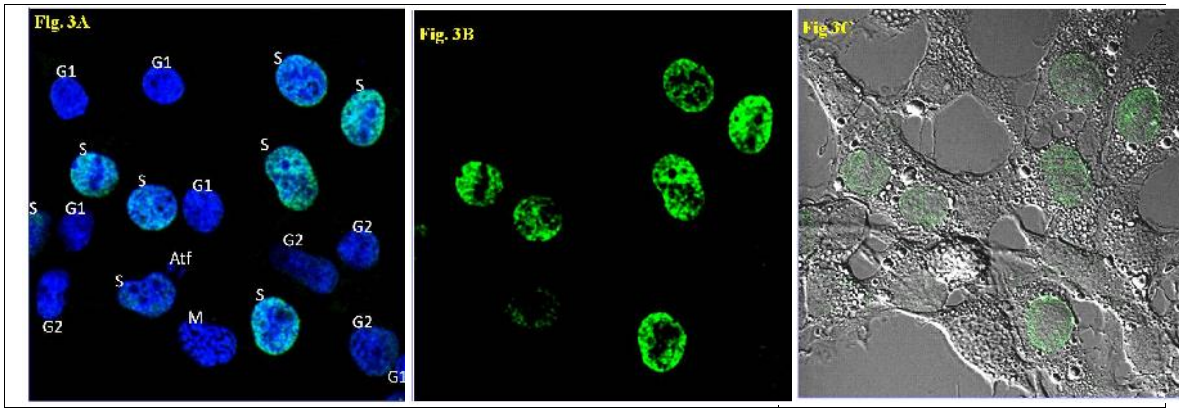

Figure 3: Various phases of the cell cycle showing same cells with different imaging approaches. A, Overlay (combined) image of both HO342 and anti-BrdU; B, Anti-BrdU for S phase determination; C, DIC image for cells in the same microscopic field for Figs. 3A and 3B

Synthetic Phase (S Phase) cells have relatively big round nuclei on a basic HO 342 staining (Figure 3A) and a flat surface with irregular outlines on DIC imaging (Figure 3C). The phase was distinct from the other phases by the positive staining with anti-BrdU (Figure 3B). Early S phase showed a rather diffuse staining with both anti-BrdU and basic HO342 staining as compared with the late S phase which showed particulate nuclei (Figures 4A and 4B).

Like the G1 phase, G2 cells did not stain with anti- BrdU (Fig. 3B) nor anti-Phospho H (Figure 4D). Unlike the G1 phase, however, G2 phase nuclei were relatively large and elongated on HO342 staining (Figure 3A) and had a bulged surface on DIC (Figure 3C). These morphological changes possibly reflect the preparation for division processes in the following M phase.

Mitosis (M phase) cells were the most distinct of all the cell cycle phases. They showed typically condensed chromatin into thread-like chromosomes (Figures 3A and 4E) on basic HO342 staining and a ballooned surface morphology (Figure 3C) on DIC imaging. The use of anti-Phospho H3 (Figure 4C) which specifically binds condensed chromosomes only during M-phase could distinguish mitotic cells from dead or damaged ones. Damaged cells also tend to round-off and hence can be confused with M-phase cells. Artifacts (Art) (Figure 3B) can also present as M phase-like particles on a basic nuclear staining but a DIC image clearly differentiates the two (Figure 3C).

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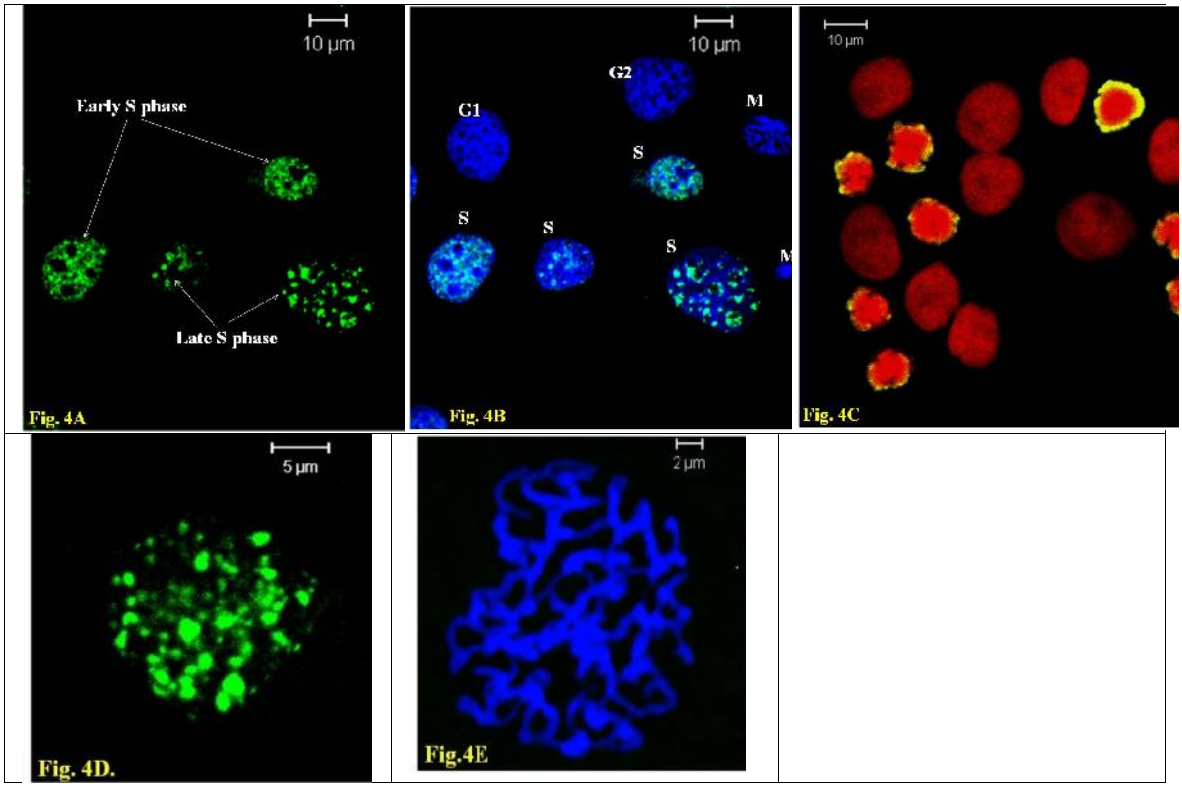

Figure 4: Immunofluorescent staining of various phases of the cell cycle. A= Anti-BrdU for S phase detection; B= Overlay (combined) image of both HO342 and anti-BrdU; C= Phospho H3 for M phase detection with Propidium Iodine counter-stain overlay; D= Anti-BrdU showing a particulate nucleus of cell in an S phase; E= HO342 stain showing condensed chromosomes of a cell in a mitotic phase

The cell cycle has been a subject of intense research due to its huge implication on cellular growth and cancer pathogenesis and therapy. This is the first study that combines several novel procedures to delineate the cells as they progress through the cell cycle. It has outlined a simple novel method of which the phase of the cell cycle for individual cells can be easily established by a combination of microscopic imaging and nuclear staining with Hoechst 33342 and anti-Bromodeoxyuridine antibody. Due to minimal perturbation and absence of protease treatments, this method models the *in situ* cellular milieu.

Previous studies in determining the individual monolayer cell morphology as they traverse through the cell cycle have largely banked on electron microscopic methods (Porter *et al.*, 1973; Lundgren & Roos, 1976) and, most recently, atomic force microscopic methods (Kelly *et al.*, 2011). Despite the diversity and the rigorous nature of cell treatments employed by these studies, the present findings agree with these previous reports. G1 phase could be detected by their small size and rounded shape. A simple basic nuclear stain like HO342, 4',6-diamidino-2-phenylindole (DAPI) or Propidium Iodide (PI) can suffice to determine the phase. Due to their morphological similarity with S phase, BrdU staining is needed to distinguish the two cyclically continuous phases. While G2 may look like any of these two phases on a basic nuclear stain, a DIC or a high resolving power phase-contrast scan can segregate this pre- mitotic phase from the former phases based on its ballooning and enlarged size (Lundgren & Roos, 1976; Toth, 1981).

G2 cells have completed the crucial process of chromatin synthesis during the previous S phase (Porter *et al.*, 1973). For preparation for the following mitotic division the cell synthesizes cytoplasmic proteins and regulatory molecules which contribute to the increased cell surface and volume. The reason for the more homogenous nuclei in G2 phase than previous phases is not clear but the completion of the chromatin synthesis during S phase could explain this interesting observation. Due to their insensitivity to BrdU detection, the two gap phases—G1 and G2—

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should be differentiated on the basis of their sizes and subtleties in their shapes. DIC imaging should be done in order to discern the actual cellular morphology.

M phase is the most distinct of all the phases of the cell division cycle. A basic HO342, DAPI or PI can easily identify the cells due to the characteristic condensed chromosomes. Although apoptotic cells and other artifacts may present as thread-like dye-absorbing particles, the use of DIC imaging combined with M-phase specific Phospho H3 antibody can easily distinguish them.

In conclusion, the major phases of the cell cycle can be differentiated easily on a monolayer culture. G1 cells are small flat and nearly perfectly spherical whereas G2 are relatively large with particulate chromatin. S-phase typically stains with anti-BrdU whereas M-phase chromatin has condensed into chromosomes. This technique is useful in segregating cells into their specific cell cycle phases. Further studies are needed to verify the usefulness in related areas such as cellular embryo cloning and cell therapy. An optimized high throughput kit need to be developed from these protocols in order to apply these findings in research and diagnostic procedures for the cell cycle study and cancer.

Declaration of no conflict of interest

The author wishes to affirm that there are no competing conflicts of interest to declare.

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