Red/green Dual Fluorescence Detection of Both the Nucleus and Nucleolus in Living Cells

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Introduction

The nucleolus represents a highly dynamic nuclear domain arising from an equilibrium between the level of ribosomal RNA synthesis and the efficiency of ribosomal RNA processing [1, 2]. Although the nucleolus is primarily associated with ribosome biogenesis, several lines of evidence now demonstrate that it has additional functions, such as regulation of mitosis, cell-cycle progression and proliferation, many forms of stress response, and biogenesis of multiple ribonucleoprotein particles. Ribosome biogenesis is regulated throughout interphase and ceases during mitosis (Figure 1). Thus, there is a direct relationship between cell growth and nucleolar activities. Nucleoli are well known to be dramatically modified in cancer cells. Additionally, a large number of key proteins from both DNA- and RNA-containing viruses are localized in the nucleolus, including the human immunodeficiency virus (HIV)-1 Rev and Tat proteins. Targeting of viral proteins to the nucleolus not only facilitates virus replication, but may also be required for pathogenic processes. The nucleolus can also be considered a sensor of stress due to the redistribution of the ribosomal proteins in the nucleoplasm through its disruption.

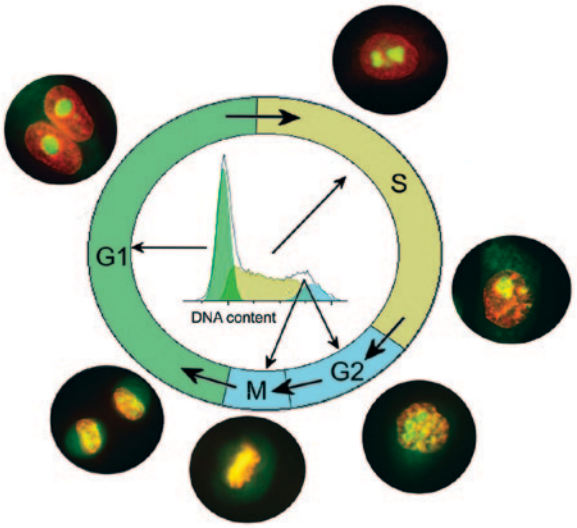

Figure 1: Changes in nuclear DNA content and nucleolar dynamics corresponding to different phases of the cell cycle, a process consisting of a series of events that leads to cell division and duplication. The cell cycle is depicted schematically along with fluorescence microscopy images of cells representing various points in the process. Cells were stained using the highlighted red/green dual fluorescence detection method. The histogram at the center of the diagram depicts cells stained with the red nuclear dye, showing DNA content distribution as determined by flow cytometry. G0/G1 and G2/M phase histogram peaks are separated by the S-phase distribution in the histogram.

Methods

Historically, nucleolar imaging approaches have required laborious and time-consuming methods, such as microinjection of fluorescently-labeled RNA, fluorescence *in situ* hybridization (FISH) or use of fluorescent protein-tagged RNA-binding proteins (i.e., GFP or YFP constructs). A novel approach for highlighting nucleoli is through use of fluorescent RNA-selective molecular probes. However, while previously available RNA probes have provided satisfactory results in fixed cells, they performed relatively poorly in live cell applications. Additionally, the principal commercially available dye for this application emits in both the green and red regions of the visible light spectrum, limiting application in multicolor fluorescence microscopy applications. Because it is difficult to study nucleolar dynamics using fixed and permeabilized cells, a fluorescent, nucleolus-selective live cell imaging dye is useful for the examination of dynamic changes in this organelle in relation to the organization of the DNA within the cell nucleus. Using a combination of a newly developed red-emitting DNA-intercalating dye, referred to as Nuclear-ID™ Red dye, and a newly developed green-emitting RNA-binding dye, referred to as Nucleolar-ID™ Green dye, we demonstrate simple dichromatic live-cell staining of the nucleus and nucleoli. The proprietary dyes exhibit minimal spectral overlap with one another (Figure 2), high signal intensity, and low photobleaching.

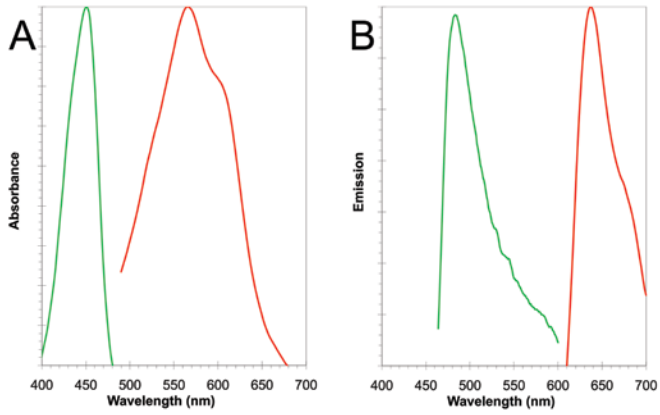

Figure 2: Spectra properties of the dyes employed in the red/green dual detection method. Absorption (A) and fluorescence emission (B) spectra of the green nucleolar dye (Abs. = 451 nm, Em = 481 nm) and red nuclear dye (Abs. = 566 nm, Em = 637 nm). All spectra were determined in 1 X phosphate-buffered saline solution. The red-emitting nuclear stain is compatible with a wide range of green-emitting fluorophores, including green fluorescent protein (GFP) and fluorescein (FITC), whereas conversely, the green-emitting nucleolar stain is compatible with a variety of orange- or red-emitting fluorophores, including yellow fluorescent protein (YFP), red fluorescent protein (RFP), phycoerythrin, and phycoerythrin-cyanine 5 dye.

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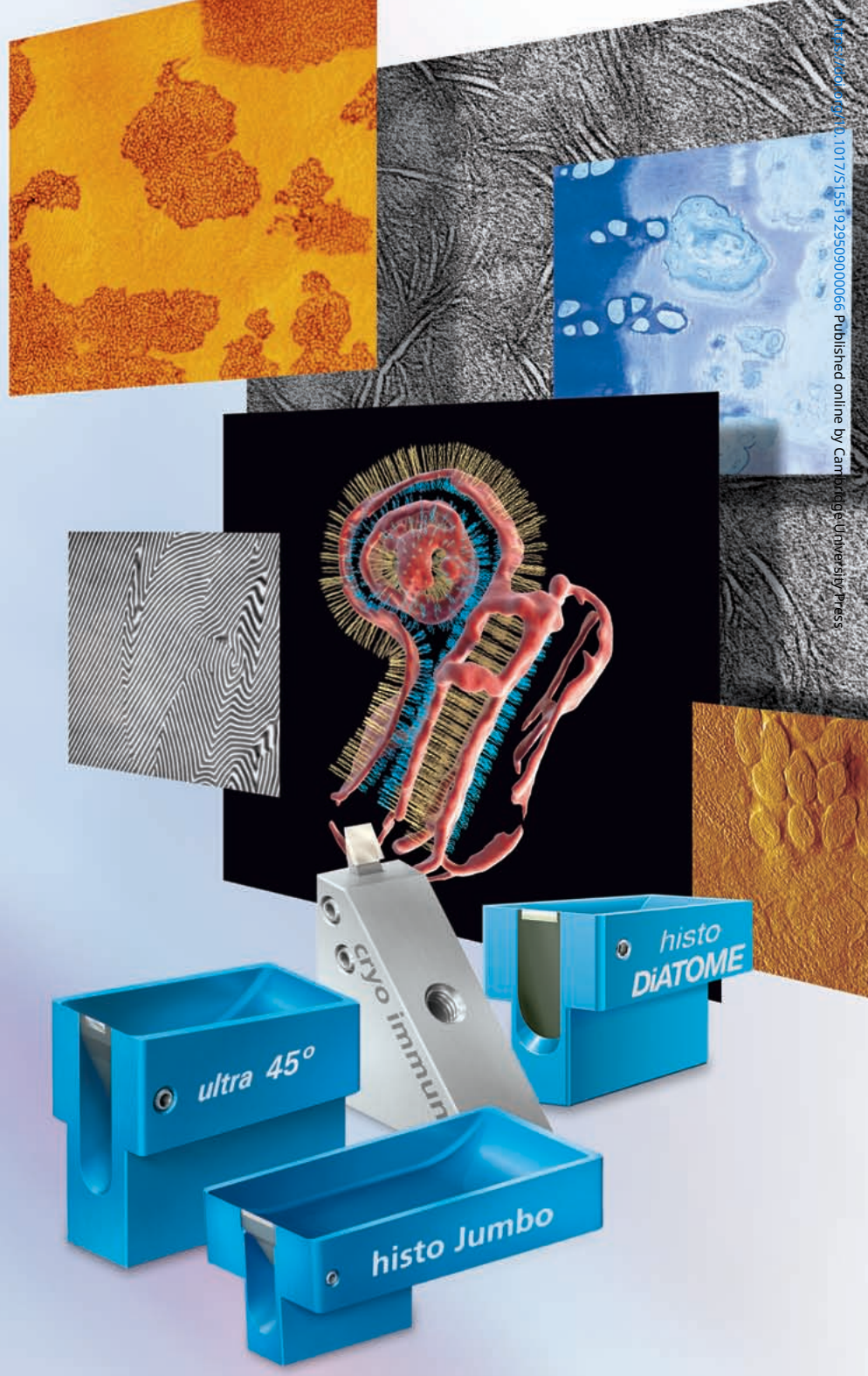

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The RNA-binding dye shows maximal fluorescence signal within the nucleoli and faint fluorescence throughout the nucleus. Weak fluorescence is also observed throughout the cytoplasm, predominantly associated with mitochondria. The red DNA intercalating dye maximally stains the DNA in the cell nucleus. Both dyes display high cellular plasma membrane and nuclear membrane permeability and are well-tolerated by living cells.

Results

Using the dichromatic live-cell staining method, we observed that the number of nucleoli in different mammalian cell types varied, and nucleoli were of different sizes, as well (Figure 3). There appeared to be an inverse relationship between the size and number of nucleoli in mammalian cells. For example, while U-2-OS human bone osteosarcoma epithelial cells were observed to contain six smaller nucleoli, HeLa human cervical carcinoma cells typically displayed two prominent nucleoli per cell, as visualized by conventional fluorescence microscopy.

Many anti-cancer drugs such as actinomycin D, adriamycin, camptothecin, cisplatin, and mitoxantrone, either localize to nucleoli or profoundly modify this organelle. In order to

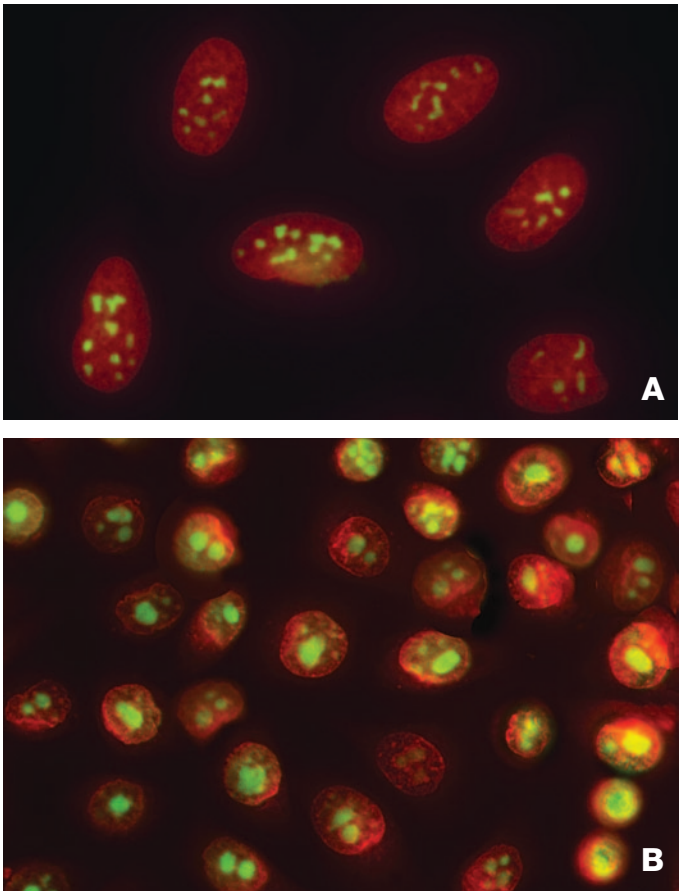

Figure 3: Live U-2-OS cells (A) and HeLa cells (B) stained using the red/green dual fluorescence detection method. The proprietary dyes exhibit minimal spectral overlap with one another, high signal intensity and low photobleaching. The green dye shows maximal fluorescence signal within the nucleoli, and faint fluorescence throughout the nucleus. Weak fluorescence is also observed throughout the cytoplasm, predominantly associated with mitochondria. The red dye maximally stains the DNA in the cell nucleus. The number of nucleoli in different mammalian cell types varied, and nucleoli were of different sizes, as well. There appeared to be an inverse relationship between the size and number of nucleoli in mammalian cells.

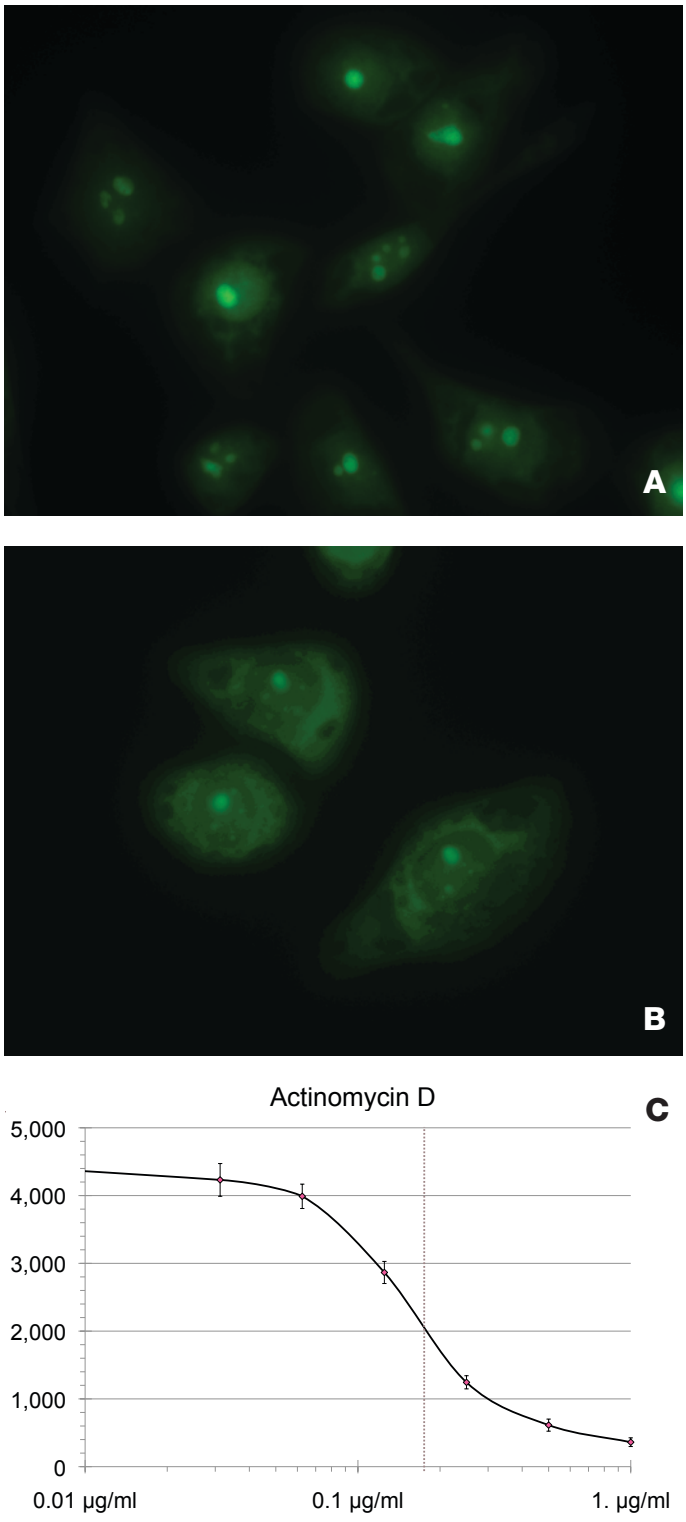

Figure 4: Actinomycin D induces a decrease in nucleolar size and number in HeLa cells. Cells stained with the green nucleolar dye show maximal fluorescence signal within the nucleoli and faint fluorescence throughout the nucleus and cytoplasm (A). When the cell is treated with low doses of actinomycin D (3 µg/ml), loss of nucleolar staining is observed (B). Using quantitative cell imaging, the half maximal effective concentration (EC50) for disruption of nucleoli by actinomycin D was determined (C).

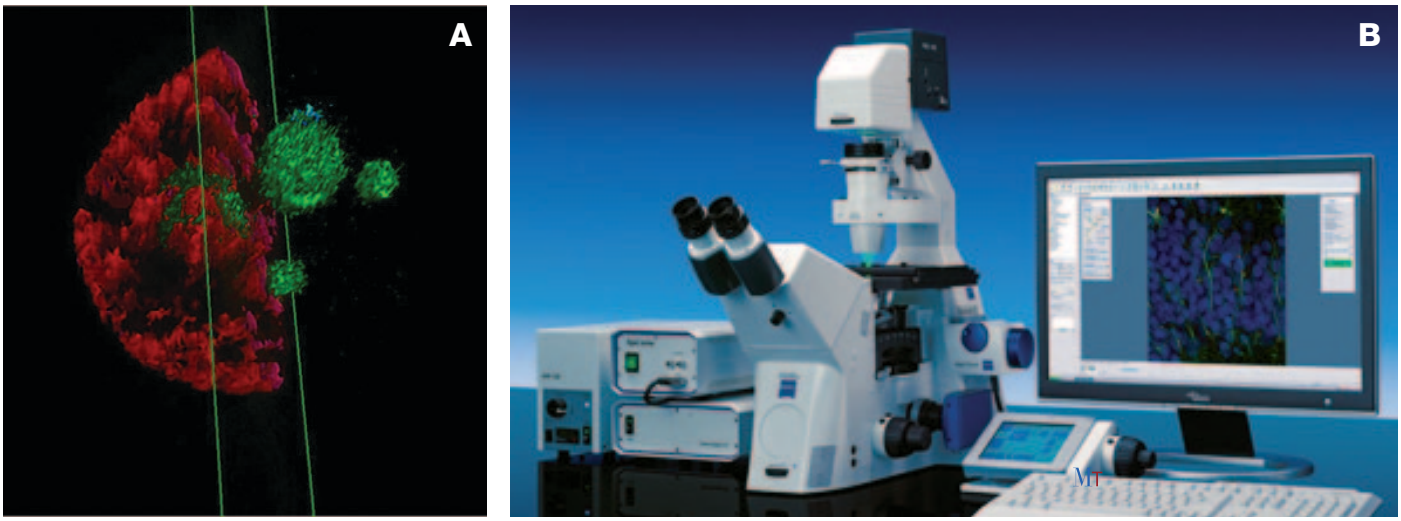

Figure 5: Three-dimensional reconstruction of the spatial relationship between the nucleoli and the nucleus using a structured illumination method (A). This was implemented with the Apotome from Carl Zeiss, Inc. (B). From a hardware perspective, the ApoTome consists of a slider, which inserts into the fluorescence beam path at the plane of the field diaphragm, and a control box to operate the slider. This imaging method enabled creation of optical sections through the nucleus using a conventional fluorescence microscope, for improved resolution along the optical axis. The optical sections were then used to create a 3-D reconstruction of the nucleus, enabling the nucleoli to be displayed in their proper spatial context relative to the body of the nucleus.

further benchmark the dichromatic staining method, changes in nucleolar dynamics were monitored after treatment with the antibiotic actinomycin D, an agent that suppresses DNA-directed RNA synthesis in vivo [3]. Typically, at higher doses of the drug (4-10 µg/ml for 4 hours), the green-fluorescence staining of the nucleolus in mammalian cells dissipated, or where still present, was dramatically reduced in amount (Figure 4A, B), while at lower concentrations (1-4 µg/ml for 4 hours), a less dramatic reduction in nucleolar staining was observed. Half maximal effective concentration (EC50) refers to the concentration of a drug that induces a response halfway between the baseline and maximum. Using quantitative imaging, the EC50 of actinomycin D was estimated (Figure 4C).

The dichromatic staining combination was found to be compatible with a broad range of fluorescence detection platforms, including conventional and confocal fluorescence microscopy, as well as flow cytometry and high-content screening (HCS) instruments. Using a conventional fluorescence microscope, a "structured illumination" method [4] was employed to create high resolution optical sections through the nucleus, facilitating three-dimensional reconstruction of the spatial relationship between the nucleoli and the nucleus (Figure 5). This demonstrated that in HeLa cells nucleoli penetrate significantly within the body of the nucleus, rather than simply being confined to its surface.

Conclusion

Potential applications for the dual detection strategy described herein include monitoring impaired ribosome biogenesis, inhibition of transcription, cell cycle dynamics and cellular stress, as well as, the distribution, trafficking and dynamics of nucleolar proteins, the distribution of viral proteins, and potentially helping to identify cancer cells. Drug toxicity screening on HCS platforms should also be feasible using the dichromatic staining approach. MT

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