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PROTOCOL

Systematic Characterization of Cell Cycle Phase-dependent Protein Dynamics and Pathway Activities by High-content Microscopy-assisted Cell Cycle Phenotyping

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Abstract Cell cycle progression is coordinated with metabolism, signaling and other complex cellular functions. The investigation of cellular processes in a cell cycle stage-dependent manner is often the subject of modern molecular and cell biological research. Cell cycle synchronization and immunostaining of cell cycle markers facilitate such analysis, but are limited in use due to unphysiological experimental stress, cell type dependence and often low flexibility. Here, we describe high-content microscopy-assisted cell cycle phenotyping (hiMAC), which integrates high-resolution cell cycle profiling of asynchronous cell populations with immunofluorescence microscopy. hiMAC is compatible with cell types from any species and allows for statistically powerful, unbiased, simultaneous analysis of protein interactions, modifications and subcellular localization at all cell cycle stages within a single sample. For illustration, we provide a hiMAC analysis pipeline tailored to study DNA damage response and genomic instability using a 3–4-day protocol, which can be adjusted to any other cell cycle stage-dependent analysis.

Introduction

Progression through the cell cycle is tightly coordinated by molecular pathways, which sense intra- and extracellular signals and orchestrate vital cellular functions with DNA replication and cell division [1]. This integrated signaling network differentially regulates activity of essential cellular processes such as DNA damage responses, transcription,

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protein biosynthesis, energy metabolism and organelle functions throughout the cell cycle [2–5].

Synchronization protocols are often used to investigate cell cycle-dependent biological processes in mammalian cells in a given experimental setting; however, these are problematic because they load experimental stresses. Immunofluorescence (IF) imaging facilitates cell cycle staging and cell phenotyping, but a combination of both strongly restricts analysis flexibility due to the limitations of fluorescence channels and antibodies, which precludes powerful applications such as cell cycle-based colocalization analysis. Live cell imaging of fluorophore-tagged cell cycle markers has been used to monitor cell cycle progression in unsynchronized cell populations, but it requires stable protein overexpression and is limited in the use of additional markers.

In order to overcome these challenges, we developed hiMAC, which integrates two-color (DAPI/EdU) cell cycle profiling and the analysis of complex cell phenotypes in a single high-content microscopy pipeline. hiMAC provides an unbiased method for cell cycle stage-dependent analysis of protein localization, interactions, structural features and pathway activities with a high statistical power, which ultimately provide cell cycle-based fingerprints for many biological processes accessible by IF imaging.

Overview of hiMAC

The hiMAC methodology enables the analysis of localization and modification dynamics of multiple proteins simultaneously in all cell cycle phases of non-synchronized cells. It consists of the integrated analysis of the cell cycle and any cell features of interest by high-content fluorescence microscopy. It can process a large number of samples, which is necessary for setups with multiple treatment conditions and time points. High-resolution cell cycle information is derived from DNA content (DAPI) and replication status (*e.g.*, incorporation of thymidine analog EdU), which confers species-independence and maximum flexibility in the choice of proteins to analyze. **Figure 1** illustrates the hiMAC workflow. A detailed step-by-step description is provided in the "Procedure" section.

Figure 2 exemplifies the hiMAC procedure for the co-localization analysis of two DNA damage markers, recombination marker Rad51 and general DNA damage marker γH2AX, throughout the cell cycle in mouse embryonic fibroblasts (MEFs). DNA lesions were induced by deletion of the DNA damage response gene *Nbs1* as verified by the immunoblotting (Figure 2A). The hiMAC protocol begins with pulse labeling of cells with EdU, followed by fixation and permeabilization (Figure 1). A click reaction is performed to label S-phase cells [6], and proteins of interest and DNA are labeled with immunostaining and DAPI, respectively. Images are recorded by automated microscopy (Figure 2B) and analyzed in a customized CellProfiler [7] pipeline to measure intensities of DAPI (DNA content and condensation) and of EdU (replication status) in individual nuclei, and to identify the pattern of objects of interest, such as proteins, organelles and micronuclei (Figure 2C). Finally, a cell cycle profile is constructed, cell cycle phases are gated (Figure 2D and E), and the objects of interest are analyzed for all individual cell cycle phases (G1, early/mid/late S and G2/M) (Figure 2F).

While hiMAC can be used to study cell cycle-dependent phenomena in general, here we specifically provide a pipeline to analyze the cell cycle phase-dependent localization of any two proteins and their interaction within the nucleus. We successfully applied this pipeline to analyze the dynamic localization of DNA damage response proteins (53BP1, Rad51, γH2AX, *etc.*) throughout the cell cycle [8].

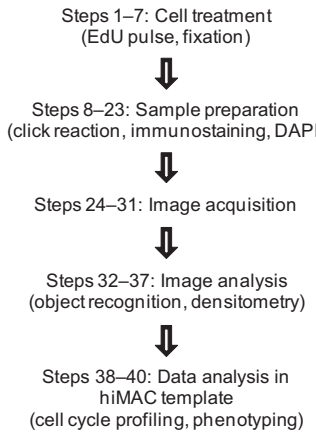

Figure 1 hiMAC workflow
The general experimental steps of the hiMAC procedure is outlined, see detailed stepwise description in the "Procedure" section.

Experimental design

Several parameters should be considered in the hiMAC protocol according to the experimental purpose. EdU pulse labeling is compatible with many cell treatments. However, EdU fails to label S-phase cells under conditions that block DNA synthesis (*e.g.*, by hydroxyurea); the EdU pulse thus needs to be applied before such a treatment. It is recommended to include one sample of asynchronous untreated cells as undisturbed cell cycle control in every experiment. Positive and negative control samples should be included for each post-translational modification to assess maximum signal induction. When quantifying the level and localization of an ectopically expressed protein, appropriate controls such as non-functional or non-degradable isoforms of the same protein, or a different protein with the same tag and similar localization should be included. The cell fixation and immunostaining methods for high antibody specificity need to be optimized before high-throughput analysis [9]. siRNA knock-down of the protein epitope of interest is a good control for antibody specificity. While non-confocal microscopy is sufficient for hiMAC cell cycle profiling, confocal image acquisition may be of greater advantage for the resolution of fine structures of interest (nuclear foci, mitochondria, *etc.*).

Results

Cell cycle profiling

Cell cycle profiling of an asynchronous population of MEF cells yielded cell cycle profiles with clear separation of G1, S and G2/M populations (Figure 2E, left panel). We next validated the stringency of cell cycle gating (**Figure 3**). Cell cycle analysis of MEFs during serum starvation and release showed the expected outcome, namely efficient G0/G1 arrest (~93%),