Detection and Visual Localization of Individual Cellular Proteins Using a Proximity Ligation Assay

Using the Cytation 5™ Cell Imaging Multi-Mode Reader to Image and Analyze Duolink® Technology

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The complexity of the proteome creates a need for more precise and accurate ways of detecting targets. Proximity ligation assays (PLA) combine the specificity of antibody based detection with a signal amplification that allows the visualization of components through fluorescence microscopy. Here we describe the use of the Cytation™ 5 Cell Imaging Multi-Mode Reader, a low cost high-value combination cell imager and microplate reader, to rapidly image and analyze tissue culture cells stained with Duolink® probes in microplates.

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Introduction

With the mapping of the human proteome the understanding of protein interactions and localization in mammalian cells grows ever more complex. The complexity of the proteome and the understanding of its inner workings create a need for more precise and accurate ways of detecting targets. Besides general detection and localization of a protein, it is necessary to investigate its interaction partners, posttranslational modifications or its role in protein complexes in order to understand the complete functional role a particular protein plays.

Proximity ligation assay (PLA) is a technology that extends the capabilities of traditional immunoassays to include direct detection of proteins, protein interactions and modifications with high specificity and sensitivity. Protein targets can be detected and localized with single molecule resolution and quantified in unmodified cells and tissues. The Duolink® In Situ reagents are based on in situ PLA®. Two primary antibodies raised in different species recognize the target antigen or antigens of interest. Species-specific secondary antibodies, called PLA probes, each with a unique short DNA strand (PLUS and MINUS) attached to it, bind to the primary antibodies. When the PLA probes are in close proximity (<40 nm), the DNA strands can interact through a subsequent addition of two other circle-forming DNA oligonucleotides. After joining of the two added oligonucleotides by enzymatic ligation, they are amplified via rolling circle amplification using a polymerase. After the amplification reaction (approx 700x) of the DNA circle has occurred, labeled complementary oligonucleotide probes illuminate the replicated product. This results in a high concentration of fluorescence in each single-molecule amplification product that is visible as a distinct bright dot when viewed with a fluorescence microscope (Figure 2).
Materials and Methods

Cell Culture

MDA-MD-175 and U-2 OS cells were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in 5% CO2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated into Corning 3904 black sided clear bottom 96-well microplates at 2,500 to 10,000 cells per well depending on the experiment.

Detection

Primary antibodies in conjunction with the Duolink® secondary PLA conjugated antibodies and the RedDuolink detection system were used to detect specific cellular targets. Mouse anti-TK-1 monoclonal antibody (cat. # WH0007083M2) was purchased from Sigma-Aldrich (St. Louis, MO), while mouse anti-elf4E monoclonal antibody (cat. #ab171091) and rabbit anti-elf4E(phospho S209) monoclonal antibody (cat. #ab76256) were obtained from Abcam® (Cambridge, MA). Duolink anti-mouse PLUS (cat.# DUO92001), anti-mouse MINUS (cat. DUO92004), anti-Rabbit MINUS (cat.# DUO92005) secondary antibodies, and the Red Detection Reagents (cat.# DUO92008), were obtained from Sigma-Aldrich (St. Louis, MO).

Imaging

Experiments were imaged using a Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT) Configured with DAPI, GFP and Texas Red light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cubes uses a 337/50 excitation filter and a 447/60 emission filter, GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the Texas Red light cube uses a 585/29 excitation and 624/40 emission filters.

Image Analysis

Multiple tiles of three-color overlaid images were digitally stitched using Gen5™ software. Typically samples were imaged by capturing a montage of images and creating a stitched composite image of wider field of view. Object cell counting of the DAPI channel was used to identify cell nuclei. Subpopulation analysis was used to determine the mean fluorescence intensity of the Texas Red channel as a means to assess TK-1 positive cells.

Results

The effect of primary antibody concentration on the PLA signal was tested. Fixed MDA-MB-175 cells were treated with various concentrations of mouse anti-thymidine kinase primary antibody and subsequently treated with Duolink® anti-mouse PLUS and MINUS secondary antibodies. Under these conditions increasing percentages of cells become positive for the presence of TK signal with increasing primary antibody until saturation is achieved. As seen in Figure 3, the percentage of TK positive cells increases with antibody concentrations up to 10 µg/ml. At this concentration the enzyme epitopes are saturated and any further increase in antibody concentration does not result in more positive cells.

Using saturating levels of primary antibody, the effect of serum concentration on thymidine kinase protein levels in U-2 OS cells was examined. Cells were seeded and allowed to attach for 16 hours in 10% serum. Cells were then switched to media containing various concentrations of serum ranging from 0.1% to 10%. As shown in Figure 4, increasing amounts of serum results in a decrease in the percentage of cells observed to be positive for thymidine kinase. Approximately 60% of the nuclei in cells treated with low (0.1%) serum are positive for TK, while less than 5% of the cells in 10% serum are positive.
Cellular localization as the result of post-translational modification of proteins can be discerned with Duolink® technology. Eukaryotic translation factor 4E (eIF4E) is a translation factor involved in the directing of ribosomes to the cap structure of mRNAs and is considered the rate limiting determinant of protein synthesis [3]. The protein is phosphorylated at position 209 and it is the phosphorylated version that is considered active [4]. Figure 5 demonstrates differences in the localization of the eIF4E from phospho-eIF4E. When U-2 OS cells are probed with a mouse primary monoclonal antibody to eIF4E, which detects all eIF4E, the protein is found distributed throughout the cytoplasm, when reacted with anti-mouse PLUS and anti-mouse MINUS Duolink secondary antibodies. However, when the same cells are probed with both a mouse anti-eIF4E and a rabbit anti-phospho-eIF4E primary antibody and treated with anti-mouse PLUS and anti-rabbit MINUS secondary antibody conjugates (See Table 1), the phosphorylated protein is observed primarily in the perinuclear region of the cytoplasm. This suggests a migration of the post-translationally modified protein to this region or that only those proteins in this region are available for modification.

Discussion

These data demonstrate that the Duolink® technology is amenable to the 96-well format. This technology is typically performed on small numbers of samples using microscope slides or coverslips as the substrate. Here we have demonstrated that large numbers of experimental samples can be assayed using microplates and more importantly the process steps can be automated as a result of using a standardized format.

Using Duolink technology in combination with image based analysis both quantitative and qualitative cellular changes can be observed. Initial experiments demonstrated the importance of epitope target saturation by the primary antibody prior to amplification. With saturating amounts of primary antibody the effect of serum concentration on the amount of thymidine kinase protein was observed to be concentration dependent. Qualitative changes in protein localization were also demonstrated using antibodies discriminating between...
elf4E and phospho-elf4E. The phosphorylated protein is found in the perinuclear portion of the cytoplasm, while the un-phosphorylated protein is observed throughout the cytoplasm.

Proximity Ligation assays such as Duolink® work very well with low copy number targets. The technology produces an amplified fluorescent signal that is physically linked to the target via antibody binding and conjugated nucleic acid amplifiers. The result is a bright spot representative of a single epitope binding event by the primary antibody. Because the cellular targets are fixed with a cross-linking agent their location within the cell can be identified.

This technology can be used to identify specific proteins within the cell or it can be used to identify specific protein-protein interactions. Duolink uses two different secondary antibody conjugates (PLUS and MINUS) that need to be in close proximity in order for the interaction of their nucleic acid conjugate tails with the oligonucleotides added as part of the ligation reaction. By using different species primary antibody pairs and their corresponding secondary antibodies, two separate epitopes, located on a single protein or located on different proteins can be identified. Only when the two different proteins themselves are in close proximity will the amplified signal be generated. If the epitopes are on separate proteins, production of PLA signal would suggest protein-protein interaction.

The EL406™ Washer Dispenser is an ideal tool to perform the assay process steps necessary prior to imaging. The EL406 provides full plate washing (96- or 384-well) using a patented Dual-Action™ manifold that has been optimized for washing loosely adherent cell monolayers. The washer dispenser also has two syringe pump and one peripump syringes for reagent dispensing. Low-cost bulk reagents, such as fixative or permeabilization buffer, can be added using the syringe pump dispensers, while more expensive reagents such as antibodies can be added using the peripump dispenser. Besides having a low dead volume, the peripump design allows for reverse flow purging of the lines that allows for the recovery of unused reagent left in the tubing lines.

The Cytation™ 5 has a number of features that enable Duolink® imaging. Four separate LED positions allow for multiplex fluorescence imaging using a number of different magnification microscope objectives. Besides identification of the protein(s) of interest, counterstaining for cytoplasmic markers or nuclei provide cellular location information. In addition the imager holds 6 objectives with magnification up to 60x. Gen5™ software provides autofocus of cells in microplates, capturing of images with both automatic or user defined parameters (LED intensity, CCD gain, integration time, etc.) and cellular analysis algorithms that allow for cell segmentation and cell counting. The Gen5 software used to control reader function is also capable of performing automated image analysis such as the counting of cells that meet fluorescence threshold and size criteria.

The melding of Duolink (PLA) detection with a standardized format, such as microplates, allows for automation being brought to bear on the sample treatment process and imaging based detection. The EL406 Washer Dispenser and the Cytation 5 Cell Imaging Multi-Mode Reader are ideal examples of automated devices to enable this technology to be run with large numbers of samples.

References

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