Application Note

Cell Analysis

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

Using the Agilent BioTek Cytation 5 cell imaging multimode reader to image and analyze Duolink technology

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Abstract

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. This application note describes the use of the Agilent BioTek Cytation 5 cell imaging multimode 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.

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, post-translational modifications or its role in protein complexes in order to understand the complete functional role a particular protein plays.

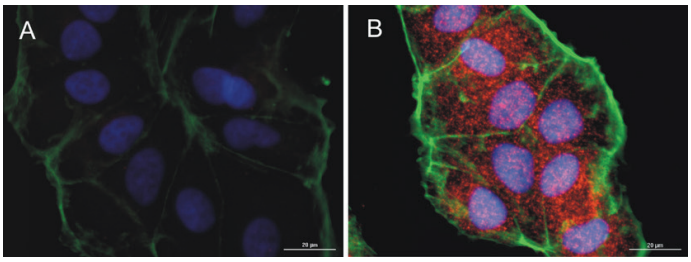

Figure 1. Specificity of Duolink staining. MDA-MB-175 cells plated in 96-well microplates and grown overnight (A) control wells where 1° antibody was omitted and (B) primary mouse anti-TK1 monoclonal antibody was added prior to subsequent processing. Cells for both figures were treated with Duolink anti-mouse PLUS and MINUS secondary antibodies and the signal amplified with the Red detection system. Cells were counterstained with the DAPI and AlexaFluor 488-phalloidin. Images were taken with a 60x objective.

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 (~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).

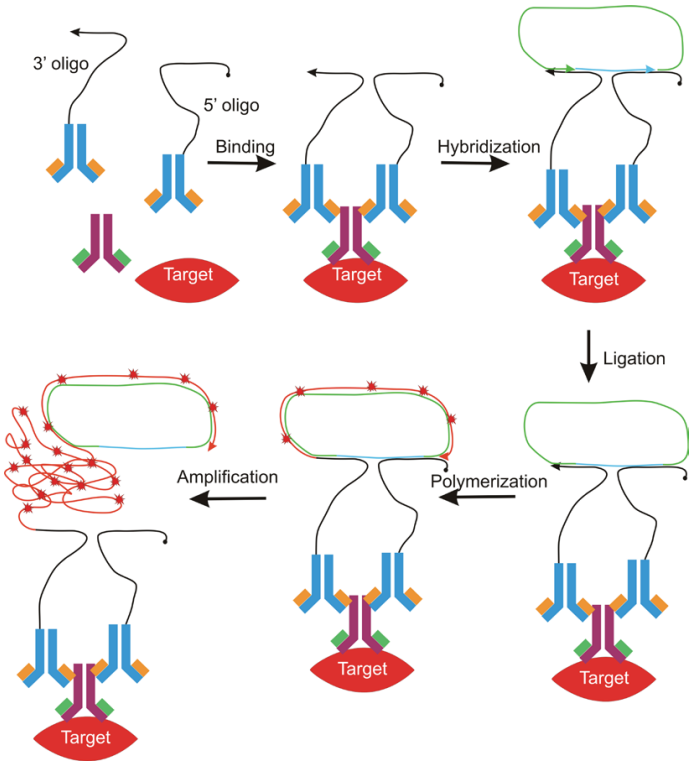

Figure 2. Duolink technology. The Duolink *in situ* reagents are based on *in situ* PLA.

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% CO_2. 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 with the Duolink secondary PLA conjugated antibodies and the Red Duolink detection system were used to detect specific cellular targets. Mouse anti-TK-1 monoclonal antibody (part number WH0007083M2) was purchased from Sigma-Aldrich (St. Louis, MO), while mouse anti-eIF4E monoclonal antibody (part number ab171091) and rabbit anti-eIF4E (phospho S209) monoclonal antibody (part number ab76256) were obtained from Abcam (Cambridge, MA). Duolink anti-mouse PLUS (part number DUO92001), anti-mouse

MINUS (part number DUO92004), anti-rabbit MINUS (part number DUO92005) secondary antibodies, and the Red detection reagents (part number DUO92008), were obtained from Sigma-Aldrich.

Imaging

Experiments were imaged using an Agilent BioTek Cytation 5 cell imaging multimode reader configured with DAPI, GFP, and Texas Red light cubes. The imager uses a combination of LED light sources in conjunction with bandpass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cubes use 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 Agilent BioTek Gen5 microplate reader and imager 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 and discussion

The effect of primary antibody concentration on the PLA signal was tested. Fixed MDA-MB-175 cells were treated with various concentrations of mouse antithymidine kinase primary antibody and then 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 shown in Figure 3, the percentage of TK positive cells increases with antibody concentrations up to10 µg/mL. At this concentration, the enzyme epitopes are saturated and any further increase in antibody concentration does not result in more positive cells.

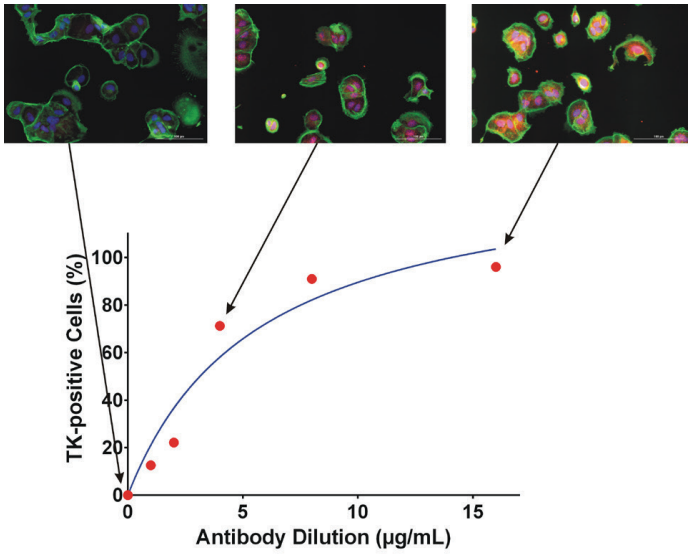

Figure 3. Effect of primary antibody concentration on Duolink signal. MDA-MB-175 cells were seeded into 96-well microplates and grown overnight at 37 °C, in a humidified 5% CO_2 environment. Cells were then fixed with 4% paraformaldehyde and assayed using a mouse anti-thymidine kinase monoclonal antibody with Duolink red detection technology. Three color montages (40x) were obtained by stitching several overlapping images. Image analysis identified cell number using object counting of DAPI stained cell nuclei. Subpopulation analysis of nuclei exceeding a threshold (11,000) for mean RFP fluorescence identified TK positive cells. Data were expressed as a percent of the total. Data points represent the mean of four determinations at each serum concentration.

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.

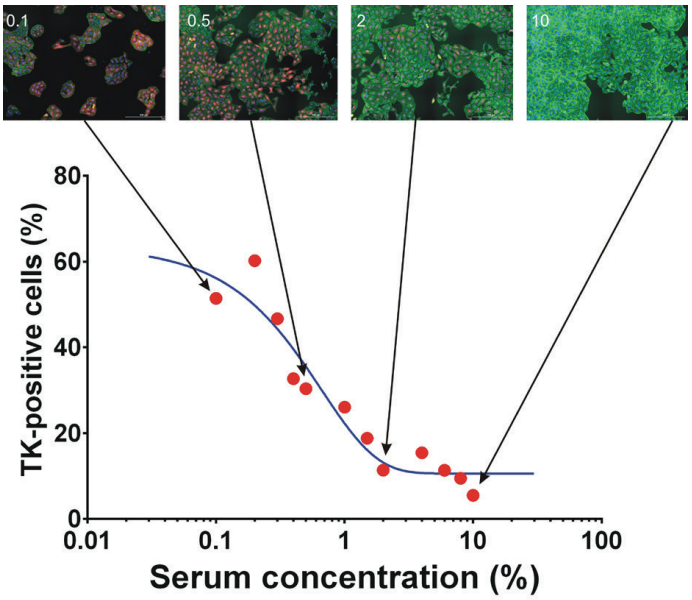

Figure 4. Effect of serum stimulation on thymidine kinase protein levels. U-2 OS cells were serum starved for 24 hours after which various concentrations of serum was added. Cells were then fixed with 4% paraformaldehyde and assayed using Duolink technology using an anti-thymidine kinase antibody. Image analysis identified cell number using object counting of DAPI stained cell nuclei. Subpopulation analysis of nuclei exceeding a threshold (20,000) for mean RFP fluorescence identified TK positive cells. Data were expressed as a percent of the total. Data points represent the mean of four determinations at each serum concentration.

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 phosph-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-phosphoeIF4E primary antibody and treated with anti-mouse PLUS and anti-rabbit MINUS secondary antibody conjugates (Table 1), the phosphorylated protein is observed primarily in the perinuclear region of the cytoplasm. This suggests a migration of the posttranslationally modified protein to this region or that only those proteins in this region are available for modification.

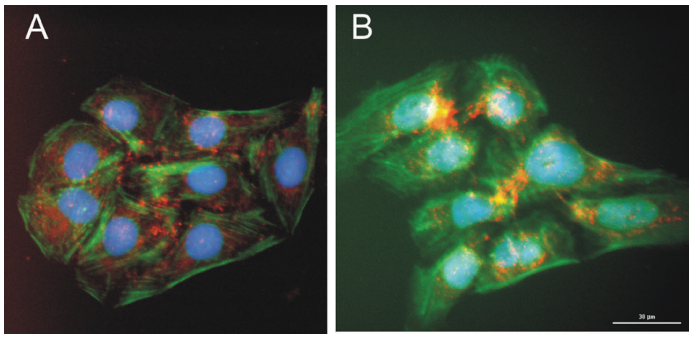

Figure 5. Localization of Total versus phospho-eIF4 protein. U-2 OS cells plated in 96-well microplates and grown overnight at 37 °C, 5% CO_2 in Advanced DMEM, supplemented with in 10% FBS serum, 2 mM glutamine. Cells were then switched to 0.1% serum and serum starved for 24 hours. Cells were then treated with 100 ng/mL EGF for 15 minutes then fixed in 4% paraformaldehyde, permeabilized, and blocked before antibody binding and Duolink processing. (A) Duolink staining using a mouse anti-IF4 monoclonal 1° antibody with anti-mouse PLUS and MINUS secondary antibodies. (B) Duolink staining using a mouse anti-eIF4 monoclonal and a rabbit antiphospho-eIF4 1° antibodies in conjunction with anti-mouse PLUS and an anti-rabbit MINUS secondary antibodies. Both reactions were treated with the Red detection system.

Table 1. Primary and secondary antibody combinations used for eIF4E studies. The species and target for the primary antibodies used to delineate eIF4E and phosphor-eIF4E localization are indicated. In addition, the species target for PLUS and MINUS Duolink secondary antibody conjugates are noted.

Target	1° Antibodies	2° Antibodies
Total eIF4E	Mouse anti-eIF4E	Anti-mouse MINUS
		Anti-mouse PLUS
Phospho-eIF4E	Mouse anti-eIF4E	Anti-mouse MINUS
	Rabbit anti-p-eIF4E	Anti-rabbit PLUS

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. This application note demonstrates that large numbers of experimental samples can be assayed using microplates and that the process steps can be automated as a result of using a standardized format.

Using Duolink technology 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 before 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 eIF4E and phospho-eIF4E. The phosphorylated protein is found in the perinuclear portion of the cytoplasm, while the unphosphorylated protein is observed throughout the cytoplasm.

Proximity ligation assays such as Duolink work 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 for the interaction of their nucleic acid conjugate tails with the oligonucleotides added as part of the ligation reaction. Using different species primary antibody pairs and their corresponding secondary antibodies, two separate epitopes, located on a single protein or 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.

Conclusion

The Agilent BioTek 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 peri-pump 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 peri-pump dispenser. Besides having a low dead volume, the peri-pump design allows for reverse flow purging of the lines that allows for the recovery of unused reagent left in the tubing lines.

The Agilent BioTek Cytation 5 cell imaging multimode reader has various features that enable Duolink imaging. Four separate LED positions allow for multiplex fluorescence imaging using several different magnification microscope objectives. Besides identification of the proteins of interest, counterstaining for cytoplasmic markers or nuclei provide cellular location information. In addition, the imager holds six objectives with magnification up to 60x. Agilent BioTek Gen5 microplate reader and imager software provides autofocusing 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 Agilent BioTek 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 automation being brought to bear on the sample treatment process and imaging-based detection. The Agilent BioTek EL406 washer dispenser and the Agilent BioTek Cytation 5 are ideal examples of automated devices to enable this technology to be run with large numbers of samples.

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