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EVENT ABSTRACT

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3D multiplex immunoplasmonics microscopy

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Introduction: Selective labeling and identification of receptors on cells can provide important clinical information, such as distinction between healthy and diseased cells, early detection and evolution of a disease, patient-specific drug selection and monitoring of the therapeutic response[1]. Immunofluorescence is the gold standard for efficient detection of antigens expressed by cells. Antibodies (Abs) conjugated to fluorescent dyes are mainly developed in the visible wavelengths and remain limited by photobleaching, high sensitivity to the environment, low light intensity, and wide absorption and emission spectra[2].
Tunable plasmonic nanoparticles (NPs) should provide higher multiplexing capacity than immunofluorescence since NPs are photostable over time, emit high light scattering at a specific wavelength (plasmon peak) and can be synthesized and functionalized with Abs[3]-[6]. The scattering peaks of silver (Ag) and gold (Au) nanospheres (NSs) are around 450 and 550 nm, respectively, and the ones from Au nanorods (AuNRs) can be extended from 600 to 2200 nm[7]. Microscopy at various wavelengths allows low illumination and fast integration times for spectral characterization of NPs in cellular environment[8]-[12]. We aim to use reflected light microscopy (RLM) for three-dimensional (3D) wide-field imaging of Abs-functionalized NPs (immunoplasmonics fNPs) targeting cell surface receptors as an alternative to immunofluorescence.

Materials: Abs anti-CD44 and anti-EGFR from abcam and Abs anti-K_V1.1 from Alomone Labs. Orthopyridyl-disulfide-poly(ethylene glycol) (5kDa)-*N*-hydroxysuccinimide (OPSS-PEG-NHS) and HS-PEG (5kDa) from Nanocs. 80 nm AgNSs from Ted Pella. 100 nm AuNSs and 40 nm x 92 nm AuNRs from Nanopartz. PBS and DAPI from Sigma-Aldrich. Secondary Abs (Alexa 488 and Cy3) from Life Technologies.

Methods: Abs were conjugated to OPSS-PEG-NHS (OPSS-PEG-Ab)[10],[13]. Citrate-capped NPs were functionalized with 0.01 OPSS-PEG-Ab/nm² and 5 µM HS-PEG (5kDa): CD44-AgNSs, EGFR-AuNSs and K_V1.1-AuNRs. The stability of fNPs was confirmed by UV-vis spectroscopy. The expression of CD44, EGFR and K_V1.1 receptors was detected by immunofluorescence and RLM on MDA-MB-231 breast cancer cells and on 661W photoreceptors. Cells were incubated for 3 h with 8 µg/mL fNPs, washed with PBS and fixed. RLM system for 3D wide-field imaging was built on an inverted Eclipse Ti microscope (Nikon) with spectral filters (500, 580 and 700 nm, Thorlabs) for fast z-scanning, optimal spectral separation and optical contrast of the fNPs in cellular environment (Fig. 1).

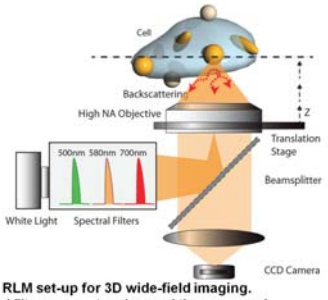

Fig. 1. RLM set-up for 3D wide-field imaging.
Spectral filters are centered around the average plasmon peak of each type of NPs in cellular environment: 500 nm for AgNSs, 580 nm for AuNSs and 700 nm for AuNRs.

Results and discussion: Deconvolution was applied to each image and treated with ImageJ to generate an image taking into account the average plasmon peak of each type of NP (Fig. 2).

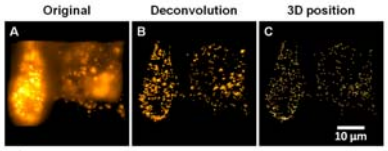

Fig. 2. Image treatment.
(A) 3D image of EGFR-AuNSs on MDA-MB-231 cells obtained with 100 nm step z-scan by RLM set-up. (B) Deconvolution of the image using experimental point-spread function. (C) 3D position of NPs obtained by local maximum filter and 3D Object Counter (ImageJ).

Immunofluorescence demonstrated the expression levels of targeted receptors (Fig. 3A-F): $CD44^+$ $EGFR^+$ $K_V1.1^+$ MDA-MB-231 and $CD44^-$ $EGFR^-$ $K_V1.1^+$ 661W. The exposure time to detect CD44 was 80 ms while it was longer for EGFR and $K_V1.1$ (500 ms). By increasing the exposure time, the background fluorescence and photobleaching become more important. This problem is solved with improved 3D identification of stable fNPs selectively labeling targeted cells by RLM (Fig. 3G-L).

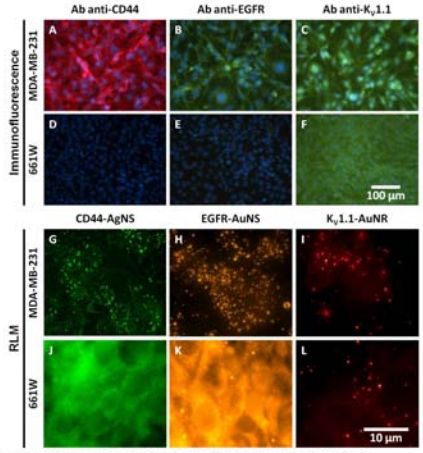

Fig. 3. Identification of CD44, EGFR and $K_V1.1$ cell surface receptors on $CD44^+$ $EGFR^+$ $K_V1.1^+$ MDA-MB-231 and $CD44^-$ $EGFR^-$ $K_V1.1^+$ 661W cells by immunofluorescence and by RLM.
(A-F) Immunofluorescence with primary Abs detected with Cy3 or Alexa Fluor 488 conjugated to IgG Abs. Cell nuclei were stained with DAPI. (G-L) RLM with CD44-AgNSs, EGFR-AuNSs and $K_V1.1$-AuNRs.

Conclusion: The developed technology is simple and compatible with standard fluorescence microscopy set-up. This technology with optical analysis of biomarkers is ready for clinical applications as an alternative to immunofluorescence.

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