

ABSTRACT

Stimulated Emission Depletion microscopy, or STED microscopy, is a technique that uses the non-linear de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction with standard confocal laser scanning microscopes and conventional far-field optical microscopes [1]. Compared to traditional confocal microscopy, STED offers exceptional improvements in resolution allowing visualization of cellular events at unprecedented levels. Rockland Immunochemicals and Leica Microsystems have jointly analyzed the sub-cellular localization of Akt using both standard confocal microscopy and high resolution STED nanoscopy. Using resting and EGF-stimulated A431 cells probed with MAb anti-Akt-(p)S473, and PAB anti-Tubulin antibodies, the STED improvement is demonstrated.

INTRODUCTION

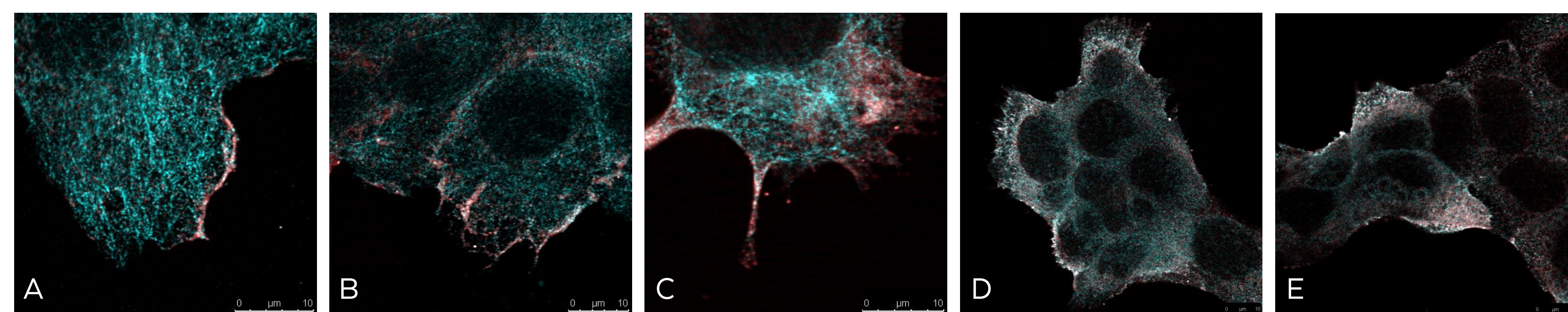
Tubulin is a ubiquitous protein and is used by cells to form microtubules. The dimers of α - and β -tubulin bind to GTP and assemble onto the (+) ends of microtubules while in the GTP-bound state. Dimers bound to GTP tend to assemble into microtubules, while dimers bound to GDP tend to fall apart; thus, this GTP cycle is essential for the dynamic instability of the microtubule. Akt is a well-studied intracellular signaling protein and is a member of the AGC-kinase family. Akt possesses a domain known as a Pleckstrin Homology domain, which binds to membrane phosphoinositides with high affinity. This is useful for control of cellular signaling because the di-phosphorylated phosphoinositide PtdIns(4,5)P₂ is only phosphorylated by the family of enzymes, PI 3-kinases (phosphoinositide 3-kinase or PI3K), and only upon receipt of chemical messengers which tell the cell to begin the growth process. Once correctly positioned at the membrane via binding of PIP₃, Akt can then be phosphorylated by its activating kinases, phosphoinositide dependent kinase 1 (PDK1) at threonine 308 and mTORC2 (at serine 473). Phosphorylation by mTORC2 stimulates the subsequent phosphorylation of Akt by PDK1. Activated Akt can then go on to activate or deactivate its myriad substrates via its kinase activity.

The Leica TCS STED (Stimulated Emission Depletion) is the first commercially available confocal microscope that enables investigation of structural details below the 90 nm resolution range. Its super resolution capacity allows imaging with a resolution two to three times higher than could ever be achieved in a conventional confocal scanning microscope[1], without the need of post-acquisition image treatments like PALM or STORM technologies. Rockland Immunochemicals and Leica Microsystems have combined resources to perform STED nanoscopy experiments using an Atto secondary antibody for the STED imaging combined with DyLight™ conjugated secondary antibodies for conventional scanning confocal. In this demonstration the microtubule protein tubulin and signal transduction protein Akt were probed for sub-cellular localization in resting and activated A431 cells.

METHODS

Conditions for STED nanoscopy: A431 cells (ATCC CRL-1555) were incubated at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, MO) with 10% FBS (Fetal Bovine Serum, Rockland Immunochemicals, Inc, PA) and with Pen Strep (Invitrogen, USA), in 75 cm² Corning CellBind cell culture flask (Sigma, MO) unless otherwise noted. When cells reached 70 to 80% confluence, they were detached using TrypLE (Invitrogen, MO), and then placed on # 1.5 mouse laminin coated coverslips in 6 wells plates for one hour at RT (Invitrogen, MO). Cells were then incubated in DMEM/FBS/Pen Strep. Once attached, the cell media was replaced by DMEM but without FBS (serum starvation step). After 15 hours of starvation, the cells were either fixed directly using 4% paraformaldehyde (PAF) for 5 minutes at room temperature (RT), or the cells were incubated with DMEM and with 100 ng/ml of Epidermal Growth Factor (EGF, Millipore, MA). Cells were then incubated in DMEM/EGF for 10, 15, or 20 minutes. Another set of cells was incubated on a coverslip with DMEM/EGF for 2 hours prior to fixation. All the incubation steps were stopped using a 4% cold PAF fixation. Once fixed, cells were rinsed in PBS at room temperature (RT). Cells were then incubated in a blocking solution (PBS/Normal Goat Serum 10%, Triton-X100 at 0.2%). This step was followed by incubation with the primary antibody mouse anti-AKT pS473 (p/n 200-301-268, Rockland Immunochemicals) at 20 μ g/ml for one hour, in blocking solution. Cells were rinsed twice in PBS for 15 minutes, and then incubated in blocking solution for 30 minutes. Atto 647N anti-Mouse IgG (Active Motif, CA) was diluted 1:1000 in blocking solution and incubated for one hour. After rinsing in PBS, the cells were incubated with α -tubulin (Millipore, CA) at 1.4 μ g/ml for one hour, rinsed and blocked as described above, then incubated in DyLight 488™ Goat anti-Rabbit IgG (p/n 611-141-122, Rockland Immunochemicals) at 1 μ g/ml for one hour. Once rinsed in PBS, the coverslips were rinsed in tap water, then mounted in 2'-Thiodiethanol (TDE) at 97%. After 12 hours at 4° C, the slides were examined using a TCS STED (Leica Microsystems, Inc.) using a 640 nm pulse laser to excite the Atto fluorescence and a Ti-Sapphire infrared multiphoton laser tuned at 750 nm as depletion laser for the detection of the Atto in STED mode. The DyLight 488 was detected using a visible Argon 488 nm laser simultaneously or sequentially with the Atto.

CONFOCAL MICROSCOPY



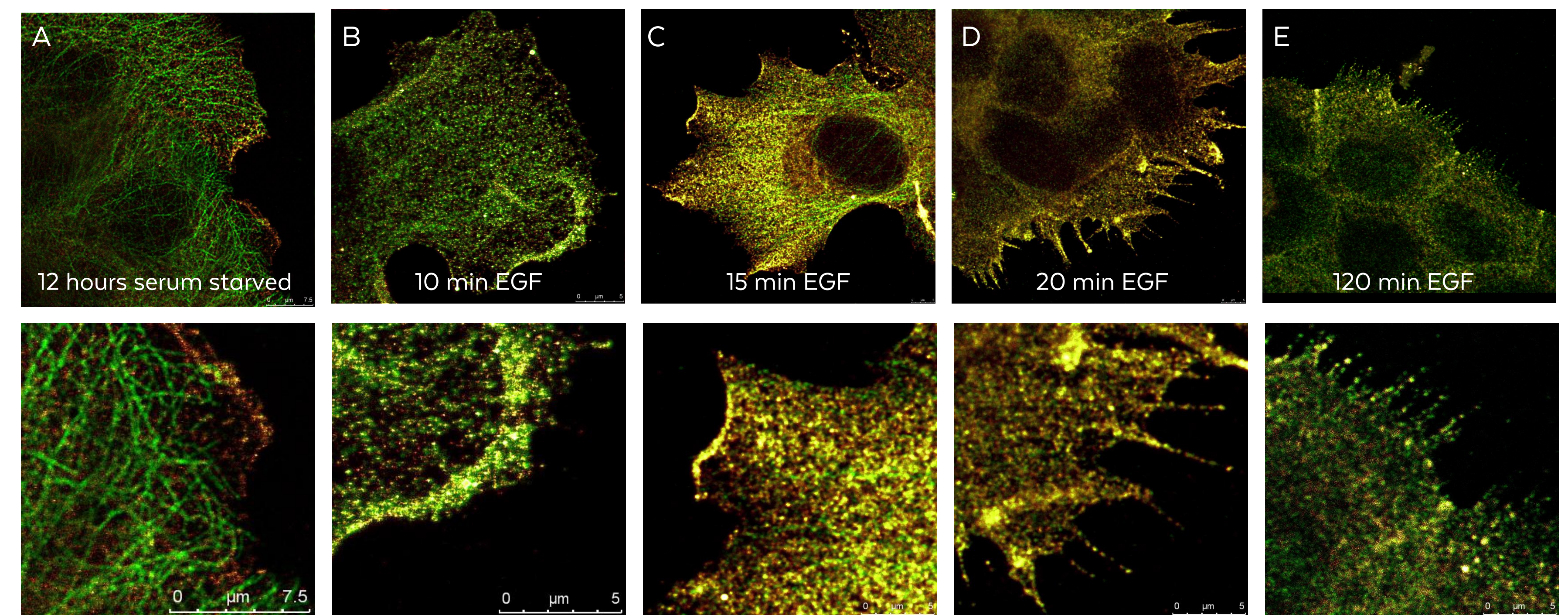
No treatment + EGF 10 min + EGF 15 min + EGF 20 min + EGF 120 min

Confocal microscopy images of EGF treated A431 cells. Confocal microscopy was used to detect the changes in Akt localization at low resolution. A Leica TCS SP5 was used to detect tubulin (cyan) stained with Rockland Immunochemicals DyLight 488™ Goat anti-Rabbit IgG, and Akt (red) stained with MAb anti-Akt pS473 and detected with atto-647N anti-Mouse IgG (Active Motif). The images show a weak diffuse staining of Akt in serum starved resting cells, and a marked activation and migration of Akt to the periphery of the cells upon stimulation with the mitogen EGF.

REFERENCES

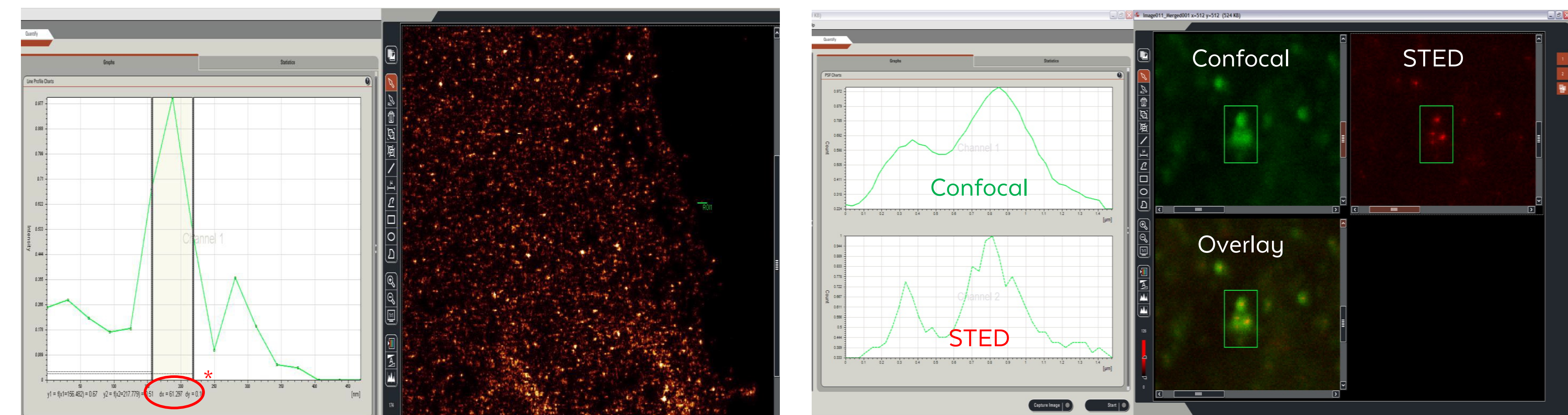
1. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Hell, S.W., et al. (1994). *Opt. Lett.* 19, 780-782.
2. The activation of Akt/PKB signaling pathway and cell survival. Song, G., Ouyang, G., Bao, S., *J. Cell. Mol. Med.*, 9:59 - 71 (2007).
3. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Franke TF, Yang SI, Chan TO, Datta K, Kazanietz A, Morrison DK, Kaplan DR, Tsichlis PN. *Cell.* 81:727-36. (1995).
4. Assays for Akt. *Methods Enzymol.* 322:400-410, Franke, T.F. (2000).

STED MICROSCOPY OF AKT & TUBULIN



Evaluation of Akt activation and migration using STED nanoscopy. A431 cells were serum starved and epidermal growth factor (EGF) was used to stimulate Akt activation. Panel A shows STED data (AKTp473, red channel) collected simultaneously with confocal signal (α -tubulin, green channel) on serum starved but unstimulated cells. Panels B,C,D,E show STED for the A431 cells stimulated with EGF for 10, 15, 20, and 120 min respectively. The arrows indicate the region shown at higher magnification (lower panels). Serum starved cells show highly organized tubulin, and very little activated Akt (mostly at the cell periphery). Upon stimulation of cells with EGF, a rapid activation of Akt is observed (panels B-C-D) along with a coinciding change in the tubulin organization, as well as an extensive cell shape change. Panel B shows cell membrane folding. Panels B, C and D show accumulation of AKT pS473 at the cell periphery. Panel C shows massive changes in AKTpS473 intensity signal with a peak of activation at 15 minutes associated with dipolymerized tubulin colocalization (yellow signal). Panel D shows after 20 min EGF stimulation phosphorylated AKT and tubulin colocalization is now limited to the cell periphery associated with newly expressed pseudo-filipodia. The total cell expression of AKTpS473 levels decreased drastically compared with the 15 min. stimulation. Panel E shows 2 hours after EGF stimulation, while the AKTpS473 level is comparable with the un-stimulated EGF cells, the microtubules are still not re-assembled or polymerized.

LEICA STED DATA EVALUATION

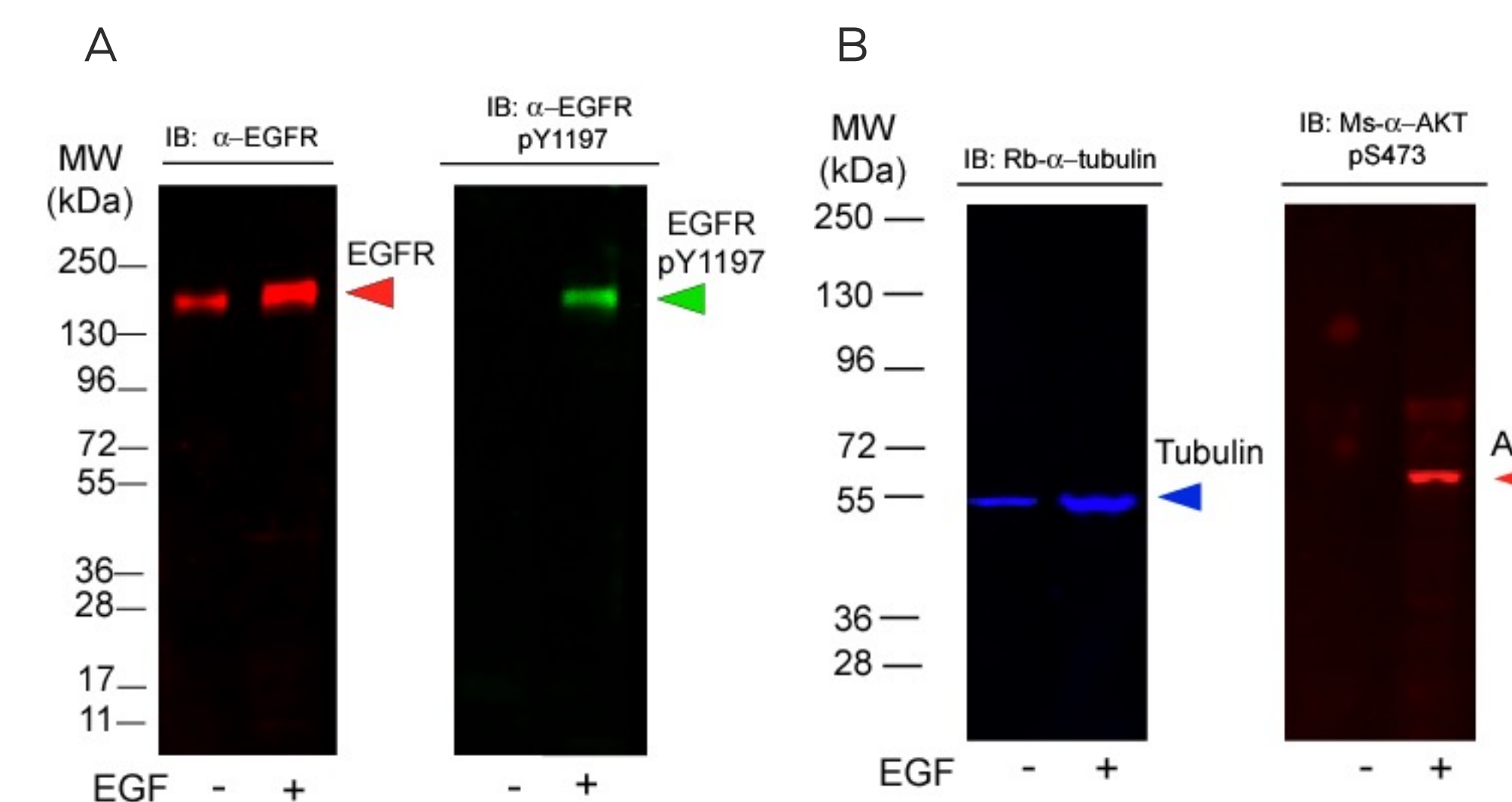


*STED resolution for the ROI = 62 nm

STED resolution compared with confocal resolution: The particle in inset image (in green) shows particles which cannot be resolved in confocal microscopy. In STED mode, (in red), the different particles are differentiated and revealed as 4 distinct particles. Left panel: Full Width Half Max peak measured in right panel -ROI(-). Particles sizes were observed to reach 300 nm or greater after 20 min post-EGF.

STED resolution after deconvolution (new AutoQuant algorithm optimized for STED Z deconvolution): The particle in inset image was measured to be 62 nm at 10 min post EGF stimulation (left panel: Full Width Half Max peak measured in right panel -ROI(-)). Particles sizes were observed to reach 300 nm or greater after 20 min post-EGF.

DETECTION OF AKT pS473 & α -TUBULIN BY WESTERN BLOT



Stimulation of EGFR pathway leads to activation of Akt. Western blotting of whole cell lysates was used to validate AKT activation. A431 cells were either unstimulated (-) or treated for 15 min with Epidermal Growth Factor (EGF) (+) to stimulate EGF receptor. All reagents were sourced from Rockland Immunochemicals. Blots were blocked with Blocking Buffer for Fluorescent Western Blotting (p/n MB-070). Blot A: Primary antibodies anti-Human Epidermal Growth Factor Receptor (EGFR) (p/n 100-401-149) and Anti-Epidermal Growth Factor Receptor (EGFR) pY1197 (p/n 600-401-928) were incubated and detected using DyLight™549 (p/n 611-142-122) (red) or DyLight™649 (p/n 611-143-122) (green) conjugated Goat-anti-Rabbit IgG. Blot B: Primary antibody against α -Tubulin (p/n 600-401-880) was detected by DyLight™ 549 Goat anti-Rabbit IgG (blue). Monoclonal antibody against AKT pS473 was directly conjugated to DyLight™649 (p/n 200-343-268) (red). Fluorescent Western data was detected using the BioRad VersaDoc MP4000 system.

CONCLUSIONS

Leica TCS STED (Stimulated Emission Depletion) confocal microscopy allows for the investigation of structural details of cells below the 90 nm resolution range as demonstrated above. STED microscopy, in direct comparison with traditional confocal microscopy, permits the assessment of protein interactions not previously visible by conventional microscopic analysis. Algorithms allow for image deconvolution and particle size determination. Antibody reactivity observed in confocal images show migration to the cell periphery upon EGF stimulation. Transient EGF stimulation is consistent with findings previously observed in lysates, therefore the migratory localization of Akt can be interpreted in context with the body of information available for Akt. Tubulin is observed proximal to Akt and may facilitate its migration upon activation. The observation of tubulin associated with Akt migration is novel and warrants further investigation. Western blotting serves a dual purpose by 1) validating the target of the antibody based on a MW assessment and the presence or absence in stimulated or unstimulated lysates and 2) shows the specificity of the antibody for activated AKT.