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Advancements in Automated Confocal Imaging

Improved image quality and quantitative analysis with water immersion objectives

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Abstract

Light microscopy is a powerful tool that provides spatial insight into biological processes. Confocal microscopy improves image quality (in terms of Z-resolution and contrast) by reducing out-of-focus light. High numerical aperture (NA) water immersion objectives further improve image quality by collecting more light (relative to lower NA air objectives of equal magnification), and better correcting for light distortion associated with refractive index (RI) mismatches between aqueous samples and the optical path of a microscope. This application note demonstrates the utility of the Agilent BioTek Cytation C10 confocal imaging reader in a nuclear translocation assay. In this assay, TGF-β1-stimulated SMAD2/3 phosphorylation and nuclear translocation are quantified in both 2D adherent and 3D spheroid cultures of A549 adenocarcinoma cells. The new water immersion capability of the Cytation C10 allows for more efficient fluorescent signal detection of this low abundance phosphorylated SMAD2/3 species, improved Z-resolution of spheroids, and compatibility with high-throughput applications, such as multireplicate dose–response curves.

Introduction

Light microscopy is a powerful tool that provides spatial insight into biological processes. By placing a pinhole in a conjugate focal plane between the sample and the light detector (camera), confocal microscopy blocks contaminating out-of-focus light, thus improving axial (Z) resolution. This improved resolution means that finer Z-sections can be taken deeper within thick samples, and even subcellular structures in adherent cells.

Image quality (in terms of Z-resolution and contrast) is negatively impacted by excited out-of-focus fluorophores, as well as transitions in the medium that emitted light must traverse when returning from the sample to the camera used for signal detection. In the latter case, how light diffracts as it travels through various mediums is described with the RI, which is a measure of how the speed of light is influenced as it passes through a material. Within an imaging system, matching the RI between the sample, mounting medium, and optical interface minimizes diffracted light, which, in turn, improves image quality (Figure 1).
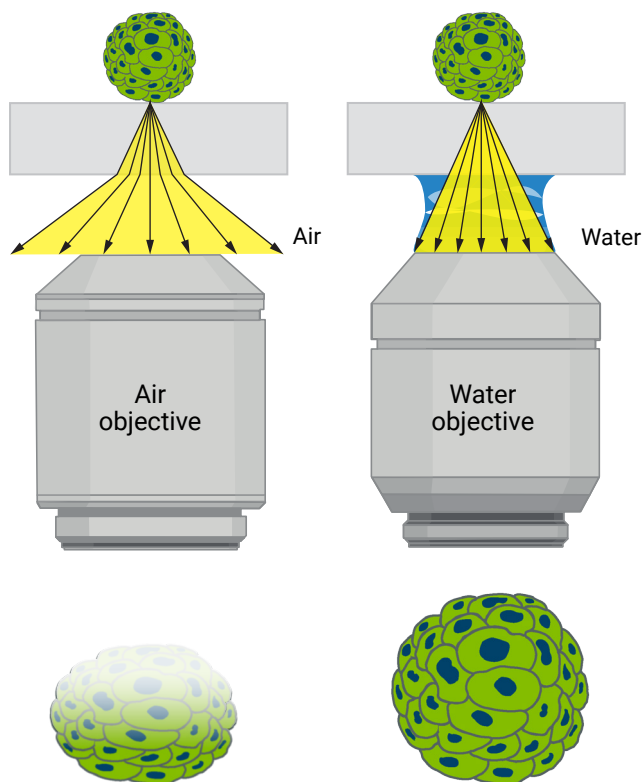

Figure 1. Water immersion objectives improve image quality. High NA and better-matched RI allows water immersion objectives to minimize Z-distortion and collect more signal (photons) with shorter exposure times, to reduce destructive sample photobleaching and improve signal detection.

In addition, at Z-planes further from the coverslip of a sample, the power of light decays exponentially, and thus excited light is dramatically reduced and detected. The NA is a measure of how much light a microscope objective can collect, such that a higher NA objective can collect more light compared to an objective with a relatively lower NA. For example, the 60x water immersion objective that is compatible with the Cytation C10 (part number 2210510) has an NA of 1.2; whereas, its air counterpart (part number 1220545) has an NA of 0.7. In practice, this allows water immersion objectives to collect equivalent signals (photons) with shorter exposure times, which, in turn, reduces destructive sample photobleaching and improves signal detection. This also means that they are ideal for dim samples that would otherwise require prolonged exposure times to collect sufficient, quantifiable signal.

The epithelial-to-mesenchymal transition (EMT) is a cellular differentiation process whereby epithelial cells lose epithelial features while acquiring mesenchymal, fibroblast-like properties, leading to reduced intercellular adhesion and increased motility. Central to stimulating EMT is the TGF-β/SMAD signaling axis, for which receptor-mediated SMAD (R-SMAD) proteins represent primary downstream effector molecules.

There are two primary SMAD signaling axes within the TGF-β superfamily. TGF-β ligand−receptor interactions result in recruitment and activation of SMAD2 and/or SMAD3 proteins, whereas bone morphogenetic protein (BMP) ligand−receptor interactions lead to recruitment and activation of SMAD1, SMAD5, or SMAD9. In either case, phosphorylated R-SMADs form a ternary complex with SMAD4; this complex then translocates to the nucleus where it regulates the expression of target genes. The ability to investigate TGF-β/SMAD signaling in detail, at multiple levels of biological complexity, is critical to gain a better understanding of the role of the EMT in cancer. In this biological context, this application note demonstrates how the Cytation C10 confocal imaging reader with water immersion capability quantitatively improves image quality while retaining high-throughput capabilities in both 2D and 3D biological samples.

Experimental

Materials

Cell lines

A549 lung epithelial carcinoma cells (part number CC-185) and HT-1080 fibrosarcoma cells (part number CCL-121) were purchased from ATCC (Manassas, VA, U.S.) and were cultured in Advanced DMEM (part number 12491; Gibco Thermo Fisher Scientific; Waltham, MA, U.S.) containing 10% FBS and 1x penicillin/streptomycin/glutamine.

Assay reagents

Human TGF-β1 recombinant protein (part number 75362) and P-SMAD2 (E8F3R) XP Rabbit mAb, which detects phosphorylated SMAD2 protein (part number 18338), were from Cell Signaling Technology ("CST"; Danvers, MA, U.S.). CF633-conjugated goat anti-rabbit (H+L) secondary antibody (part number 20123) was used to detect anti-P-SMAD2 and was from Biotium, Inc. (Fremont, CA, U.S.). Hoechst 34580 (part number H21486) and Alexa Fluor 488 phalloidin (part number A12379) were from Thermo Fisher Scientific (Waltham, MA, U.S.). Propyl gallate (antifade reagent used for imaging; part number 02370) was from MilliporeSigma (St. Louis, MO, U.S.).

Growth factor treatment

Agilent 96-well imaging microplates (part number 204626-100) were treated with 10 µg/mL fibronectin (part number F1141; Sigma-Aldrich, Burlington, MA, U.S.) diluted in DPBS for 30 minutes, followed by three washes with DPBS before cell seeding. Seeded, adherent cells, or 2,000-cell spheroids formed in ultralow attachment (ULA), round-bottom, 96-well microplates (part number 4520; Corning, NY, U.S.), were serum-starved for 18 hours in basal culture medium lacking FBS (A-DMEM). Cells were then treated for 60 minutes at 37 °C with human TGF-β1 recombinant protein at the indicated concentrations.

Sample preparation for 1 µm TetraSpeck calibration microspheres

A volume of 1 µm TetraSpeck beads (part number T7282; Thermo Fisher Scientific; Waltham, MA, U.S.) was diluted 1:100 in 100 µL ice cold 2% Type I collagen (part number 354236; Corning, NY, U.S.) and added to wells of an Agilent 96-well imaging microplate. The plate was then plated in a tissue culture incubator set to 37 °C, and the collagen was allowed to solidify for 1 hour. Once the collagen was completely solidified, wells were filled with PBS containing 0.2% propyl gallate, then imaged.

Sample preparation for adherent cells

Adherent cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes, followed by one wash with DPBS containing 0.5 M glycine. Cells were permeabilized with 0.5% Triton X-100 for 5 minutes, then blocked for 30 minutes with 5% BSA. Cells were then incubated overnight at 4 °C with anti-P-SMAD2 Rabbit monoclonal antibody diluted in 5% BSA containing 0.1% Tween 20 (according to supplier recommendations). After three washes with PBS + 0.1% Tween 20, cells were incubated for 1 hour with goat anti-rabbit polyclonal antibody diluted to 1:1000 in 5% BSA containing 0.1% Tween 20, 5 µM Hoechst 34580, and 1x Alexa Fluor 488-conjugated phalloidin. Following three washes with PBS + 0.1% Tween 20, wells were filled with PBS containing 0.2% propyl gallate, then imaged.

Sample preparation for spheroids

TGF-β1-treated spheroids were transferred from the ULA 96-well microplates that they were formed in to an ULA 24-well microplate (part number 3473; Corning, NY, U.S.). Here, they were washed three times with DPBS before fixation in 4% PFA for 1 hour at room temperature, followed by one wash with DPBS containing 0.5 M glycine. Spheroids were then permeabilized with 0.5% Triton X-100 for 1 hour, followed by an overnight block with 5% BSA at 4 °C. Spheroids were then incubated overnight at 4 °C in anti-P-SMAD2 rabbit monoclonal antibody, diluted in 5% BSA + 0.1% Tween 20. Spheroids were then transferred to 6-well culture plates where they underwent five 30-minute washes with PBS + 0.1% Tween 20. Samples were transferred to fresh wells of an ULA 24-well microplate and incubated with goat anti-rabbit polyclonal antibody diluted to 1:1000 in 5% BSA containing 0.1% Tween 20, 5 µM Hoechst 34580, and 1x Alexa Fluor 488-conjugated phalloidin diluted in 5% BSA + 0.1% Tween 20, followed by hourly washes with PBS + 0.1% Tween 20. Spheroids were then transferred to 6-well culture plates where they underwent five 30-minute washes with PBS + 0.1% Tween 20, then a final wash with PBS (no Tween 20). Spheroids were then immobilized in the bottom of Agilent 96-well imaging microplates (part number 204626-100) by being submerged in 100 µL of 2% collagen. Once the collagen solidified, wells were filled with PBS containing 0.2% propyl gallate, then imaged.

Gen5 imaging, image processing, and cellular analysis

Imaging and image processing

Using the Agilent BioTek Cytation C10 confocal imaging reader and a 60x 1.2 NA water immersion objective in confocal mode (60 µm pinhole disk), Z-stacks of adherent cell samples or spheroids (1 and 0.6 µm step sizes respectively) were acquired in three channels: DAPI (Hoechst 34580), GFP (AF488-conjugated phalloidin), and CY5 (CF633-conjugated goat anti-rabbit secondary antibody, used to visualize phosphorylated SMAD2/3), with the DAPI channel used to set Z-focus height using laser autofocus. Maximum intensity projections of multichannel Z-stacks underwent a background-reduction transformation step before cellular analysis. For Figures 3 and 4, Z-stack OME-TIFFs acquired in Agilent BioTek Gen5 software were imported into ImageJ (FIJI), where XZ images were generated.

Cellular analysis

Primary masks of nuclei were established using Hoechst 34580 signal (DAPI channel; yellow outlines in images), and integrated phosphorylated SMAD2/3 or SMAD4 nuclear signal of individual nuclei was quantified using the CY5 channel as a secondary mask. The reported phosphorylated SMAD2/3 signal was derived by first adjusting for the nuclear area using the following custom metric formula: [Area-corrected Integral signal] = (Integrated CY5 signal) ± (Nuclear area).

The area-corrected integral phosphorylated SMAD2/3 signal measured at each TGF-β1 concentration was converted to, and reported as a fold change of the signal response relative to vehicle control using the following custom data reduction step: [Nuclear P-SMAD2/3 fold change] = (Area-corrected integrated P-SMAD2/3 signal) ± (Mean area-corrected integrated P-SMAD2/3 signal of vehicle control).

Results and discussion

Water immersion imaging enables gentler imaging by reducing light exposure

The NA is a measure of how much light a microscope objective can collect, such that a higher NA objective can collect more light compared to an objective with a relatively lower NA of equal magnification. This allows water immersion objectives to collect equivalent signals with fewer exposure times, which, in turn, reduces destructive sample photobleaching. This can be demonstrated in 2D cell cultures where changes in nuclear signal (P-SMAD2/3) are quantified after exposure to 100 ng/mL TGF-β1 (Figure 2). Imaging the same sample using a 60x 0.7 NA air objective with identical exposure settings reveals a severely undersampled image (Figure 2B).

To obtain an image with a comparable signal in each respective channel using an air objective, exposure times needed to be increased 3.3 fold for DAPI, 8 fold for GFP, and 2.4 fold for CY5 (Figure 2C).
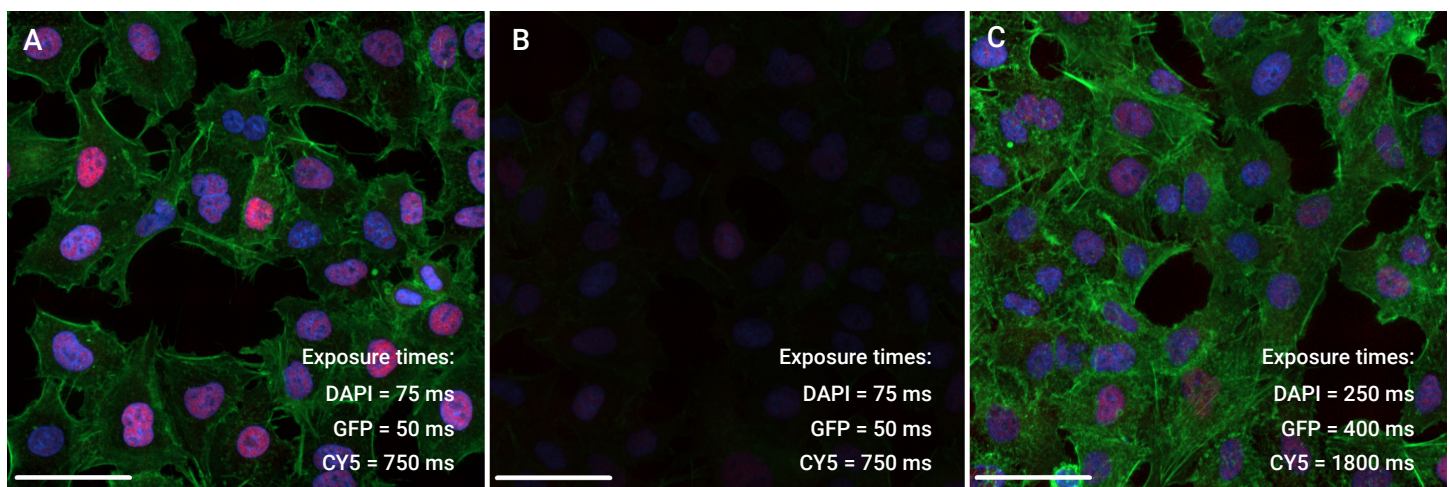

Figure 2. Water immersion imaging enables gentler imaging by reducing light exposure. Adherent A549 cells seeded in an Agilent 96-well microplate, treated with TGF-β1, stained for nucleus (Hoechst 34580), F-actin (Alexa Fluor 488 phalloidin), or phosphorylated SMAD2/3 (CF633 goat anti-rabbit). Imaged with either an (A) 60x 1.2 NA water immersion objective, (B) 60x 0.7 NA air objective with identical exposure settings to (A), or (C) 60x 0.7 NA air objective with exposure settings adjusted to approximately match signal intensities to those obtained in (A). Scale bar = 100 µm.

Water immersion imaging preserves the fluorescent signal and reduces Z-distortion at greater Z-depths

Shorter working distances (the distance an objective can travel toward a sample before making direct contact) typically accompany higher NA objectives, which is the case with the water immersion objectives available with the Cytation 10. Also, due to better RI matching with aqueous samples, water immersion objectives dramatically improve image quality in two important ways: 1) signal intensity is better preserved throughout the imageable Z-range, and 2) image distortion in the Z-axis is greatly reduced. This can be objectively evaluated using uniformly shaped objects with stable fluorescence and consistent dimensions, such as TetraSpeck calibration microspheres (Figure 3). A Z-stack of microspheres colloidally immobilized in a collagen matrix that spans the Z-range of

a 60x 1.2 NA water immersion objective working distance (280 µm) was captured in the GFP channel with either a 60x air objective (Figure 3A) or 60x water immersion objective (Figure 3B). The Z-stack was then translated into the XZ orientation so that the Z-depth could be visualized. Compared to the 60x air objective, the 60x water immersion objective dramatically retains signal intensity and reduces Z-distortion at greater Z-depths. Individual microsphere examples are highlighted at the furthest Z-depths (Figure 3C and D), or at a Z-depth closest to the well bottom (Figure 3E and F). This improved image quality at greater Z-depths can be observed in XZ images of HT-1080 spheroids in all three channels imaged: CY5 (Figure 4A and B), DAPI (Figure 4C and D), and GFP (Figure 4E and F).
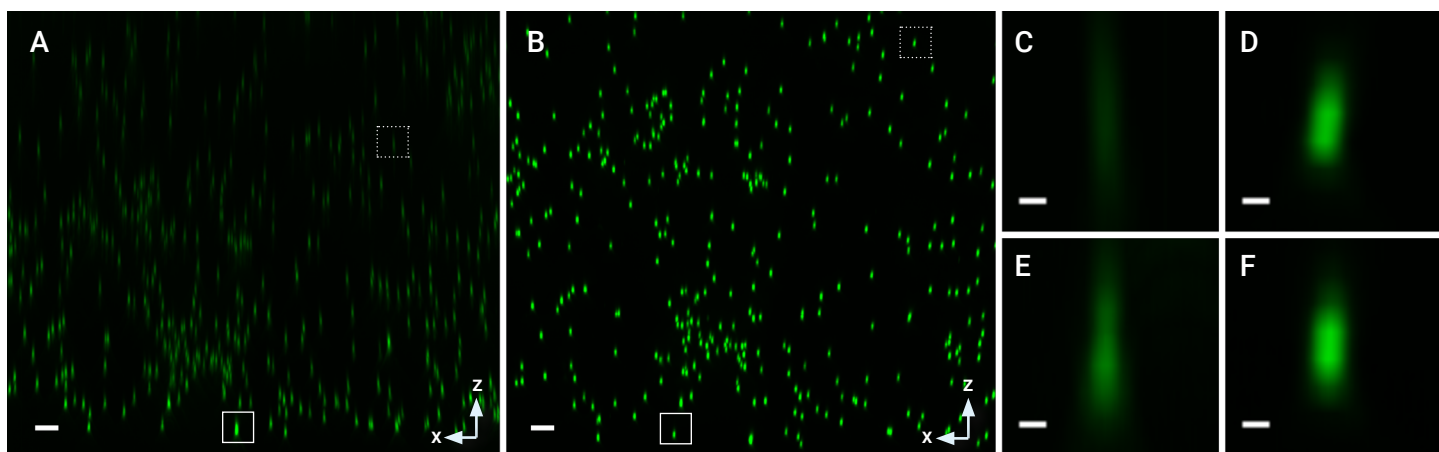

Figure 3. Water immersion imaging preserves fluorescent signal and reduces Z-distortion at greater Z-depths. XZ view of 1 µm TetraSpeck microspheres colloidally immobilized in a collagen matrix within an Agilent 96-well microplate. Z-stacks were acquired using either a (A) 60x 0.7 NA air objective or (B) 60x 1.2 NA water immersion objective. Scale bar = 10 µm (X-axis), while the total Z-height represents an approximate 234 µm depth. (A-B) The fluorescent signal decay and Z-distortion using an air objective is apparent with a microsphere near the furthest Z-range (A,B: dotted-line box) from (C,D) the bottom of the well and, to a lesser degree, a (E,F) microsphere closest to the well bottom (A,B: solid-line box). Scale bar = 2 µm (X-axis).
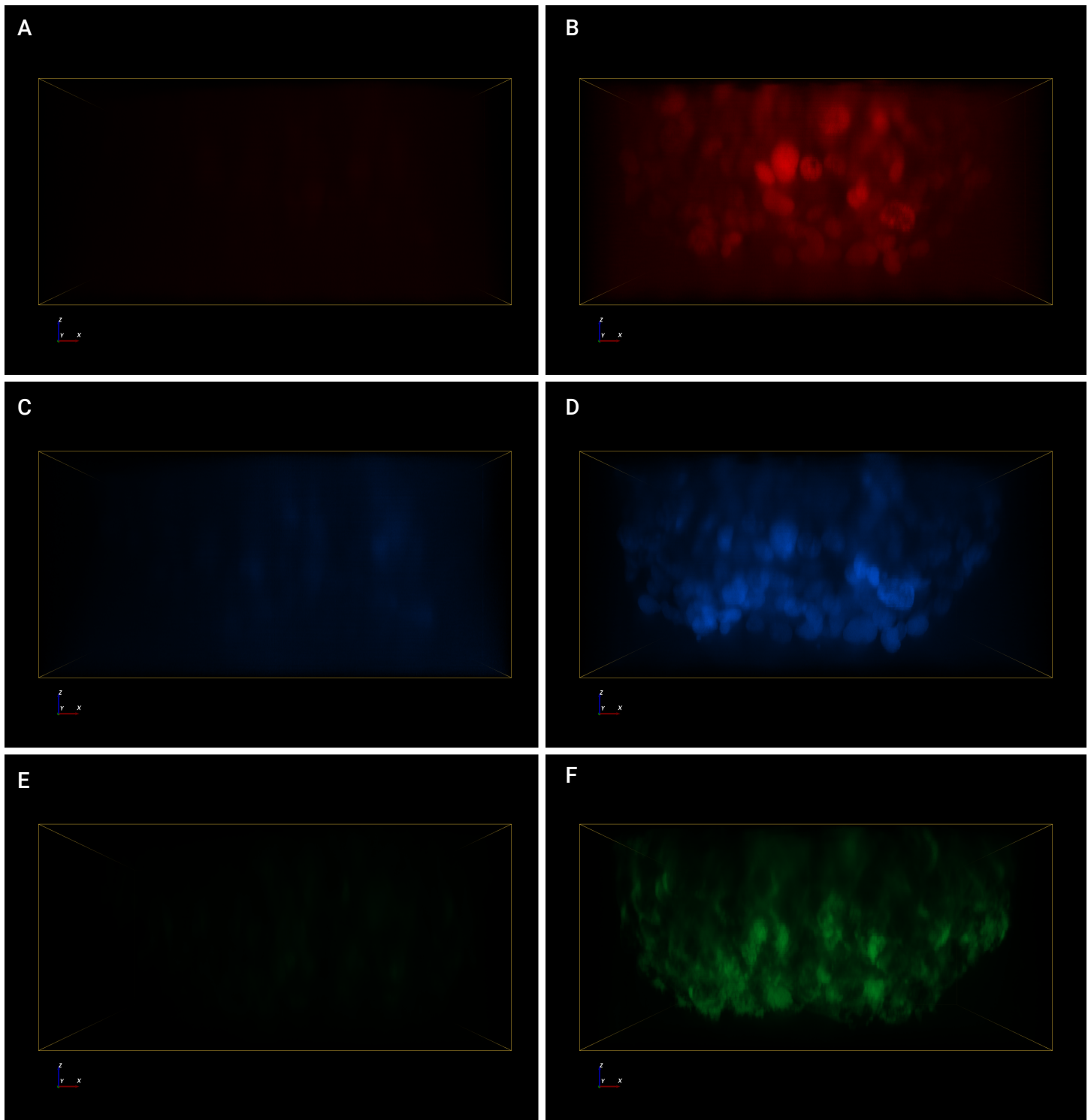
Figure 4. Water immersion imaging preserves fluorescent signal and reduces Z-distortion at greater Z-depths. XZ view of an HT-1080 spheroid Z-stack imaged with either (A,C,E) a 60x 0.7 NA air objective or (B,D,F) a 60x 1.2 NA water immersion generated using the Agilent BioTek Gen5 software 3D viewer. Acquisition settings (exposure times) were kept consistent across the air and water immersion objectives for the respective channels. While fluorescent signals were faint with the air objective, preserved fluorescent signal and reduced Z-distortion can be observed with the water immersion objective in the (A,B) CY5 channel, (C,D) DAPI channel, and (E,F) GFP channel. Scale bar = 50 µm (X-axis).

Cytation C10 water immersion capability enables quantitative analysis of multicellular biological samples

The water immersion capability of the Cytation C10 retains compatilibity with analytical features equipped in Gen5 software. HT-1080 spheroids treated with vehicle control (Figure 5A) or 100 ng/mL TGF-β1 (Figure 5B) were immunostained for phosphorylated SMAD2/3 (red) and counterstained with Alexa Fluor 488-conjugated phalloidin (green) and Hoechst 34580 (blue). Z-stacks of spheroids were then acquired using either a 60x 0.7NA air or 60x 1.2NA water immersion objective with 1μm step sizes. Due to the field-of-view constraints of 60x objectives and the size of the spheroid, the first 100 μm was imaged, and maximum intensity projections of the full stack were subsequently generated (Figure 5A and B). For quantitative analysis, maximum intensity projections spanning the first 30 μm underwent a cellular analysis step where nuclei were identified as a primary mask (Figure 5C, white outlines), and phosphorylated SMAD2/3 signal (CY5 channel) was quantified as a secondary channel.
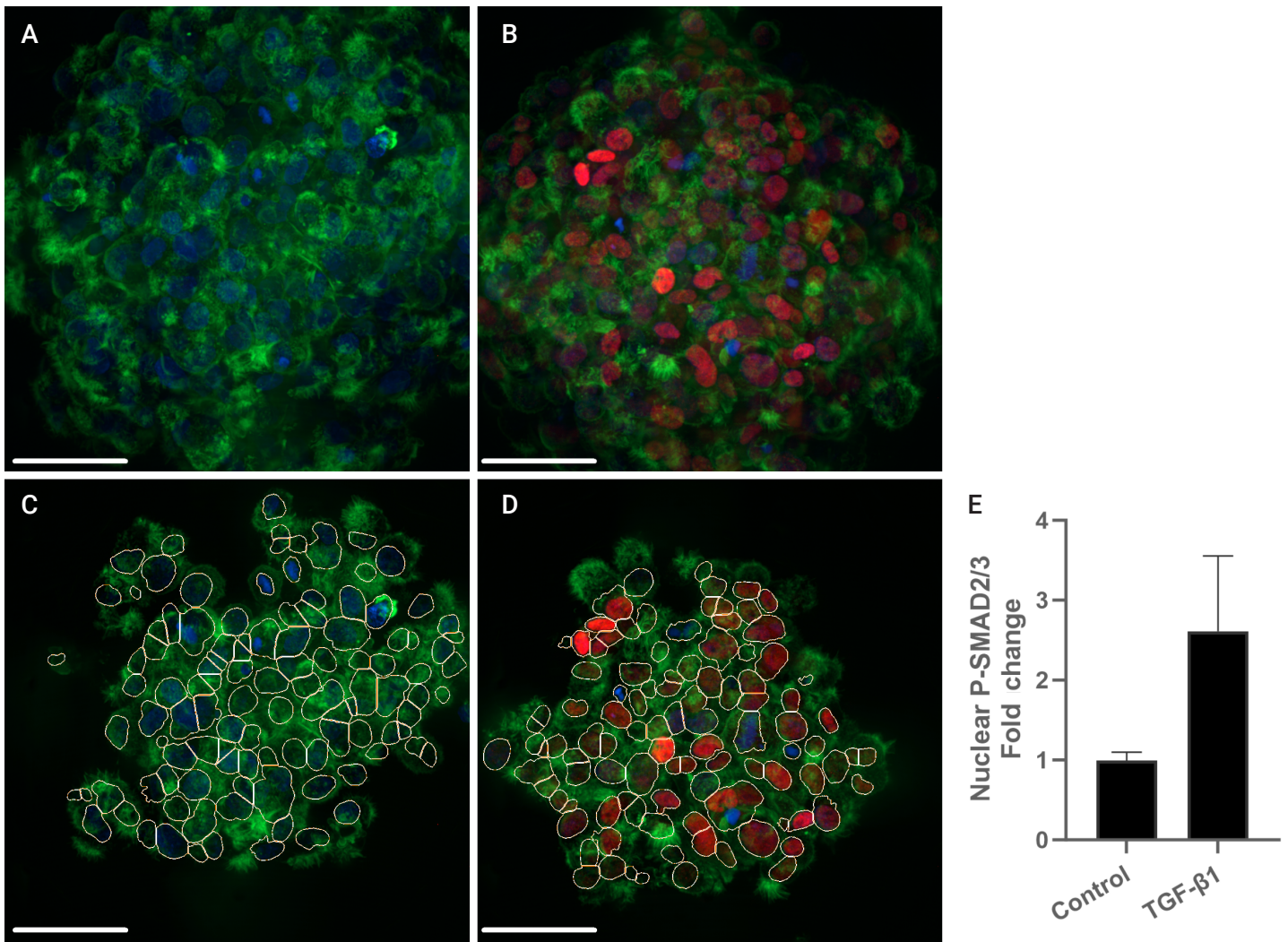

Figure 5. Agilent BioTek Gen5 software cellular analysis in water immersion. HT-1080 spheroids treated with (A) control or (B) 100 ng/mL TGF-β1 were imaged to a depth of 100 μm, followed by a background reduction and maximum intensity projection of the full Z-stack. Using Gen5 software, nuclei within the first 30 μm of spheroids treated with (C) control or (D) 100 ng/mL TGF-β1 were identified in maximum intensity projections and nuclear phosphorylated SMAD2/3 signal (red) was quantified (E). Scale bar = 50 μm.

Cytation C10 enables high-throughput imaging applications with water immersion capability

The ability to maneuver across vast XY positions while maintaining a column of immersion media is a major obstacle when working with conventional immersion objective systems. However, the Agilent BioTek water immersion system overcomes this challenge by flushing a sufficient bolus of water over the objective and maintaining that bolus on the fly as imaging progresses across a multiwell microplate. This feature allows the signature high-throughput imaging capabilities of the Cytation C10 confocal imaging reader to be retained with water immersion objectives in a microplate format (Figure 6A). This is demonstrated in both adherent cell cultures (Figure 6B) or individual spheroids (Figure 6C) imaged in a 96-well imaging microplate. Because water immersion is integrated into the Cytation C10, high-throughput confocal imaging is capable in both sample types, which enables dose−response values to be derived (Figure 6B), or biological replicates be imaged so that statistically robust results can be reported (Figure 6C).
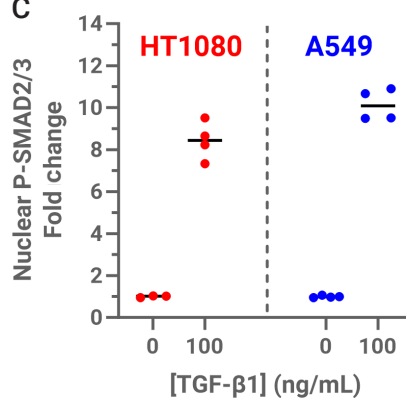
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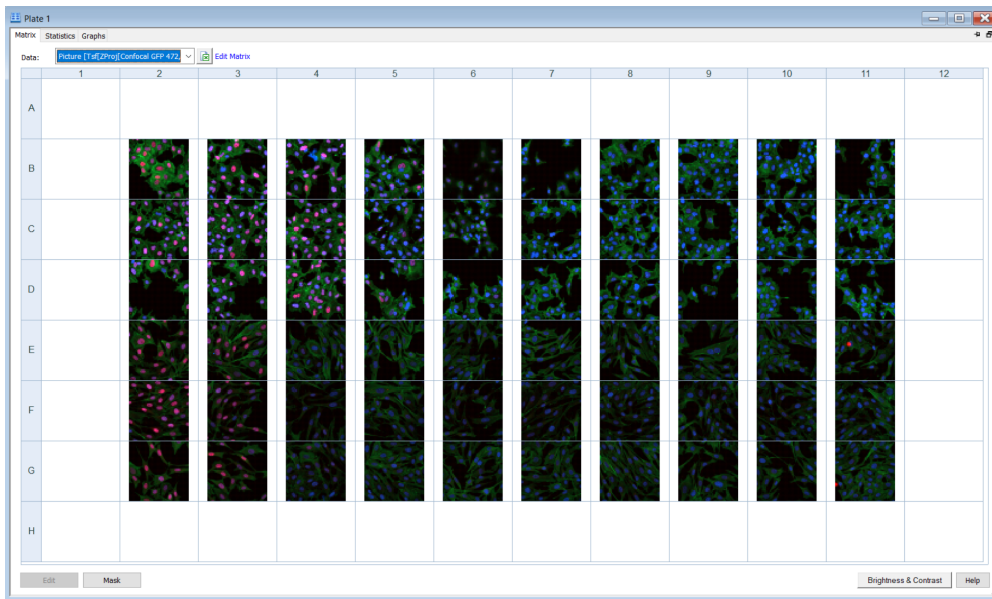
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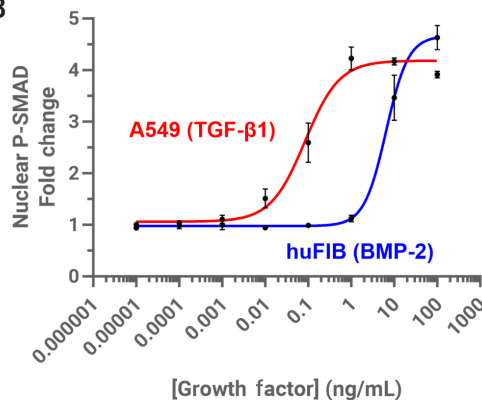
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Figure 6. The Agilent BioTek Cytation C10 confocal imaging reader enables high-throughput imaging with water immersion capabilities. (A) Matrix view of two-dimensional adherent cells seeded in an Agilent 96-well microplate (rows B to D = A549; rows E to G = huFIB) were imaged in one run with a 60x 1.2 NA water immersion objective. (B) A dose−response curve for either TGF-β1 (A549) or BMP-2 (huFIB) was derived. (C) Single HT-1080 or A549 spheroids were imaged in 96-well microplates with a 60x 1.2 NA water immersion objective in a single run (images not shown), cell analyses were conducted, and a fold change of nuclear phosphorylated SMAD2/3 was calculated.

Conclusion

Confocal microscopy improves image quality (in terms of Z-resolution and contrast) by reducing out-of-focus light. High NA water immersion objectives further improve image quality by collecting more light (relative to lower numerical aperture air objectives of equal magnification), and better correcting for light distortion associated with RI mismatches between aqueous samples and the optical path of a microscope. Using 2D and 3D cellular approaches that assay TGF-β/SMAD signaling, this application note demonstrates that the water immersion capability of the Agilent BioTek Cytation C10 confocal imaging reader improves image quality in three important ways: 1) it enables gentler imaging by minimizing light exposure; 2) it reduces Z-distortion of 3D biological samples; and 3) it is conducive with high-throughput applications. The enhanced confocal capabilities of the Cytation C10 with water immersion unlock advanced imaging potential to better understand biological processes.

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