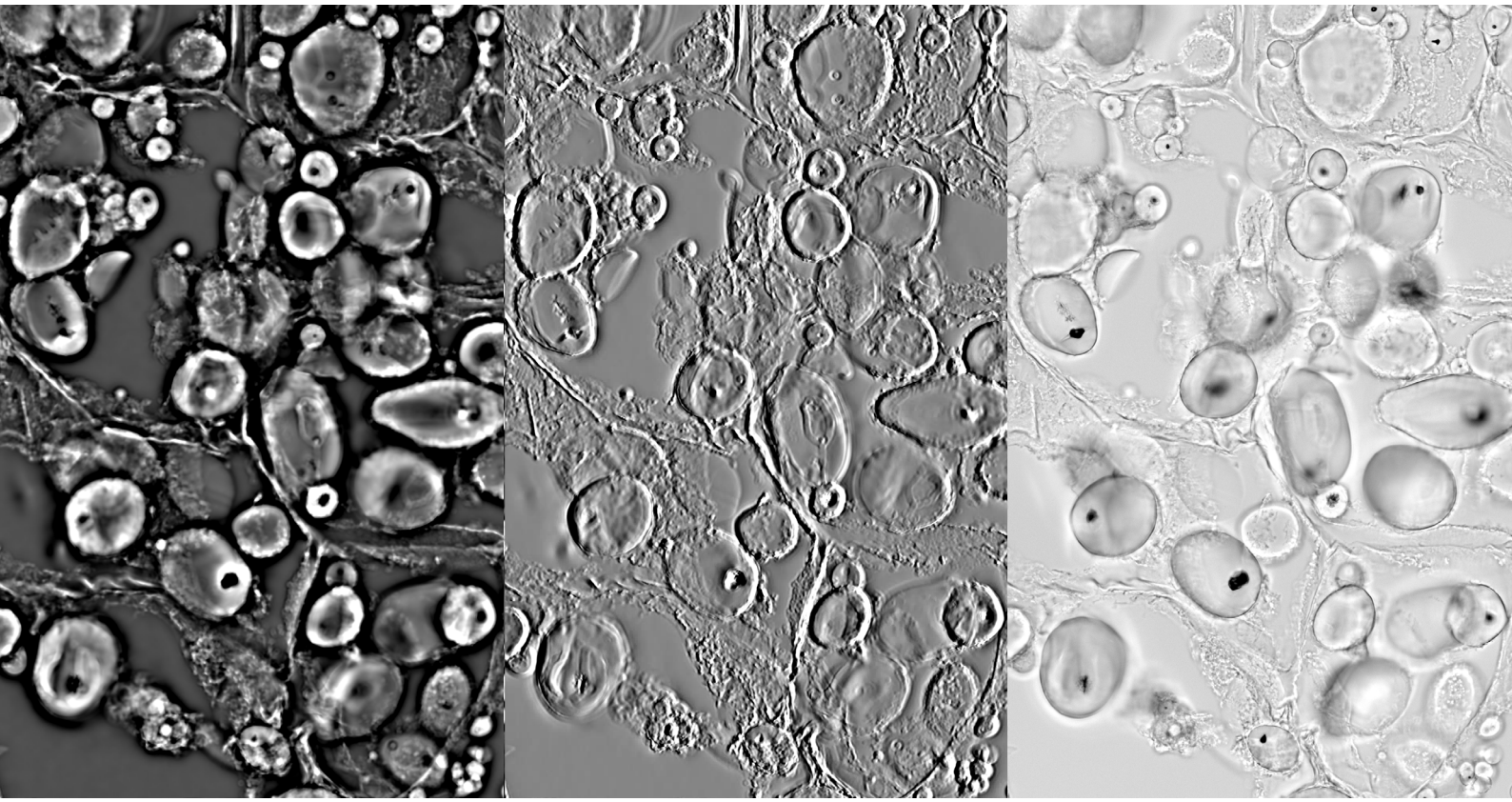
Transport of Intensity Equation (TIE)

A New Brightfield Method for Imaging and Fast Autofocusing During Automated Slide Scanning with ZEISS Axioscan 7



ZEISS

Seeing beyond

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The use of focus maps as an autofocus technique for high-throughput, whole slide imaging experiments is widely adopted but has many disadvantages when applied to fluorescently labeled samples. ZEISS has implemented a new brightfield contrast technique, Transport of Intensity Equation (TIE), with the ZEISS Axioscan 7 digital slide scanner. TIE overcomes previous challenges providing fast and reproducible autofocusing without compromising fluorescence imaging quality as well as offering additional contextual data.

Introduction

Efficient scanning for whole slide imaging of tissue sections requires high-throughput, automated protocols. These methods yield higher levels of data collection for more thorough analyses and lower hands-on requirements which improves time management of laboratory researchers and staff. However, the success of these experiments relies on the imaging system's ability to accurately and consistently autofocus as it collects hundreds to thousands of images.

One commonly used method for autofocusing is the use of a focus map, which is created by pre-scanning the selected area, sampling axial focus points, and using an algorithm to automatically determine the axial position with the sharpest image. This method works well with brightfield illumination and chromogenically stained slides; however, this method has disadvantages when moving to fluorescently labeled samples.

Figure 1 *ZEISS Axioscan 7*

With fluorescently labeled tissues, a fluorescent counterstain, such as DAPI, which labels all cell nuclei that are typically distributed throughout the tissue section, can be used to generate a focus map. However, repeated DAPI imaging can cause bleaching, particularly with sensitive fluorescent labels typically used with multiplex experiments. Additionally, using fluorescence illumination to find the focal point often takes longer compared to using brightfield illumination and, when working with hundreds to thousands of images, the length of the experiment can become unmanageable. One might consider using brightfield illumination with contrast enhancing methods, such as phase contrast or DIC. However, these methods require additional optical elements in the light path: matching phase rings in the condenser and objective for phase contrast or Wollaston prisms and analyzers for DIC. Not only do these components add to the overall cost and complexity of the imaging system, but their presence in the light path negatively impacts the quality of fluorescence imaging.

To address these challenges, ZEISS has implemented the Transport of Intensity Equation (TIE) contrast enhancing method with the digital slide scanner ZEISS Axioscan 7. This brightfield technique avoids bleaching by using the transmitted light source and does not reduce the fluorescence imaging performance as no additional optical elements are introduced into the fluorescence light path. Now the advantages of fast sample detection and focus mapping can be combined with the highest quality fluorescence imaging for challenging whole slide imaging experiments, including those using multiplexing or other sensitive fluorescent labels.

Overview of the Transport of Intensity Equation (TIE)

TIE creates digital contrast images by acquiring three images in transmitted light brightfield while closing the aperture of the condenser completely to create an almost coherent bundle of light. The three images are each separated by one depth of field (DOF) in the axial direction: one image is from the plane of focus, one from above, and one from below, as shown in Figure 2. The raw images are then processed, resulting in either a phase contrast image or a relief contrast image, reminiscent of differential interference contrast (DIC).

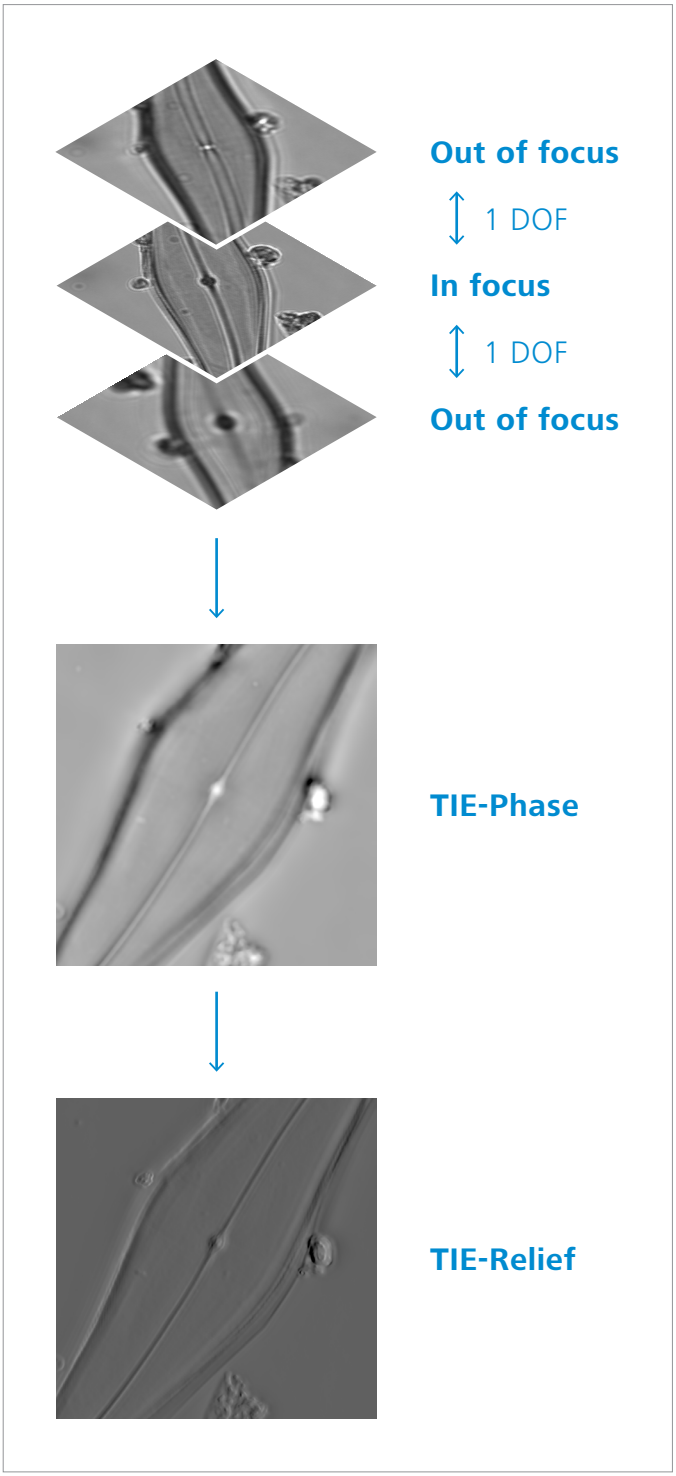

Figure 2 *Working principle of TIE contrast. Phase contrast or relief contrast are two visual representations of TIE.*

TIE can be used to determine the sample focus quickly and reliably for thin, transparent samples, as shown in Figure 3. A focus stack is acquired quickly using the ZEISS Axioscan 7 flash illumination method followed by a software calculation for image sharpness for each focus plane.

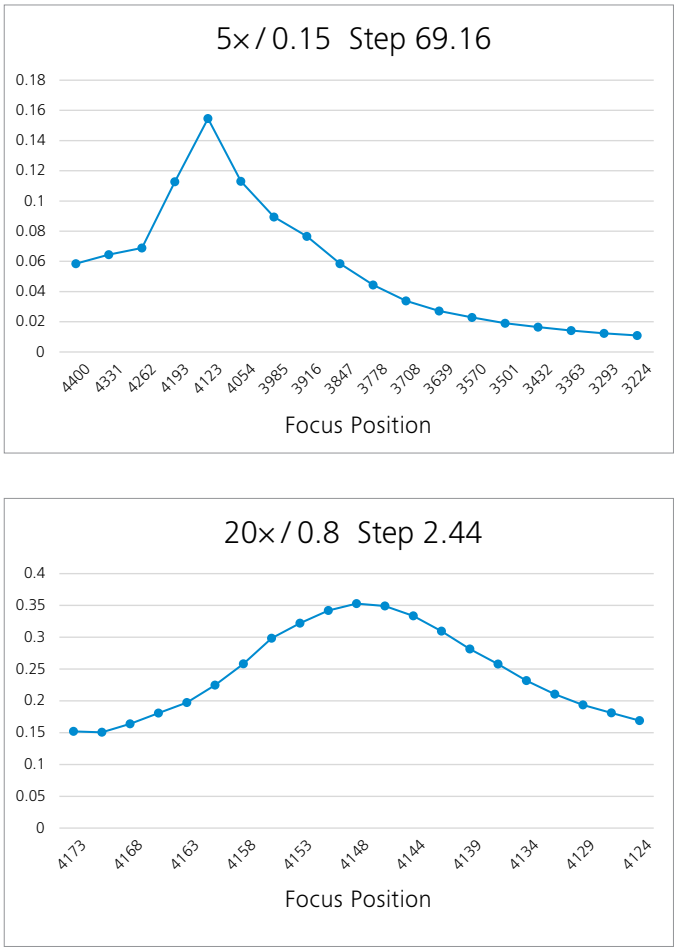

Figure 3 *Results for one focus support point determined in the coarse focus (top) and the fine focus map (bottom) creation results when using TIE contrast. X-axis: focus position. Y-axis: result of focus merit (image sharpness) calculation.*

TIE for Fast and Efficient Autofocusing

A major advantage of the implementation of TIE with ZEISS Axioscan is the ability to use it for fast and efficient autofocusing with the rapid acquisition method using flashed illumination.[1] Very brief pulses of illumination from a very bright white light LED are synchronized with the camera acquisition while moving the focus axis in a continuous fashion. In typical scenarios, acquiring a full stack followed by the calculation will not take more than a few seconds per focal point.

This speed increase is significantly higher over traditional focus determination using the fluorescent DAPI channel, which is typically ~20 seconds. Additionally, TIE also avoids photodamage to fluorescent dyes in the sample as well as the undesired artifacts of darker tiles for the DAPI channel during samples acquisition. Such artifacts can also cause problems for automated image analysis.

While TIE is ideally used for very thin translucent samples with low contrast, such as monolayer cell cultures (< 3 µm) or thin tissue sections (< 5 µm), it has also been shown to be useful when scanning thicker (> 5 µm) tissue sections.

TIE Provides Additional Contextual Information during Data Collection

As a main transmitted light contrast method with ZEISS Axioscan 7, TIE adds the ability to create phase contrast and relief contrast images during data collection. This provides contextual information, particularly with fluorescence experiments. This is demonstrated in Figure 4. Mouse kidney tissue was stained with DAPI and Alexa 647 (Phalloidin). TIE provides a nice phase contrast, which can be combined with the fluorescence information to show where the nuclei and actin structures are positioned in relationship to the glomeruli.

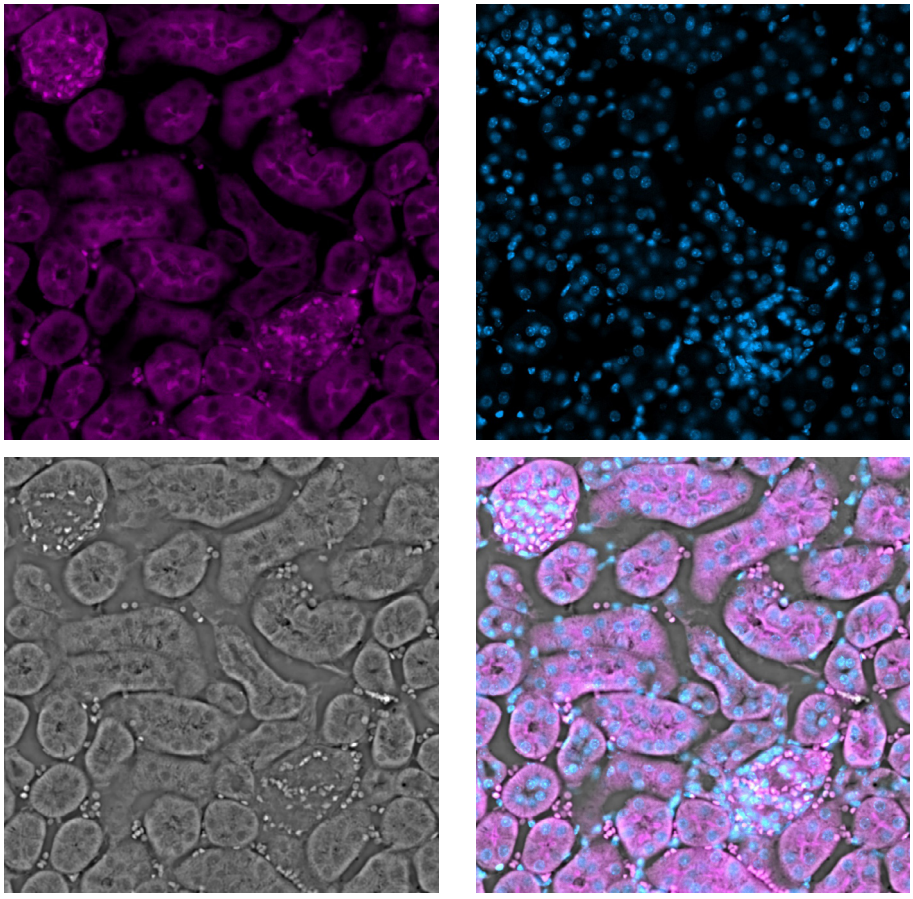

Figure 4 *Mouse kidney tissue section stained with DAPI and Alexa 647 (Phalloidin). A) DAPI fluorescence, B) Phalloidin Alexa 647 fluorescence, C) TIE-Phase contrast, D) Merge fluorescence and TIE Phase. Image courtesy of Katarzyna Dobaczewska, Microscopy and Histology Core Facility, La Jolla Institute for Immunology, San Diego, USA.*

Conclusion

To meet the need for efficient autofocusing for automated, whole slide imaging of fluorescently labeled samples that does not cause photobleaching or reduce image quality, ZEISS has implemented TIE as a novel brightfield contrast method with the ZEISS Axioscan 7 digital slide scanner. Not only does TIE meet these needs, it can also be used to provide additional structural context to fluorescence data.

TIE is a valuable addition to ZEISS Axioscan 7, a high-performance slide scanner with unsurpassed fluorescence imaging quality for high-throughput, complex experiments.

Supplementary Information: Theoretical Background

The phase information of the propagated light wave becomes visible as intensity difference via interference. There are numerous techniques reviewed in the literature to image the phase properties of a sample, ranging from classical phase contrast[2] and holographic methods[3] to illumination diversification[4] and wavefront sensors[5]. TIE is built on the fact that phase information becomes visible after some propagation in free space because of interference of best focus light with the change of the light field in the defocused images. In a paraxial approximation for coherent illumination, this change is reduced to a gradient and leads to the following transport of intensity equation (TIE)

$$\frac{\partial}{\partial z} I = -\frac{1}{k} \nabla \left(I \, \nabla \varphi \right)$$

first discovered by Teague[6], which relates the gradient of the phase ϕ to the change of intensity along the optical axis $\frac{\partial}{\partial z} I$. Here k is the wavelength dependent wave number and I is the best focus intensity.

By recording focal stacks, ZEISS Axioscan 7 measures the intensity in several planes. The best contrast is obtained by reducing the aperture of the condenser to a very small value, e.g., to 0.07 NA. We approximate $\frac{\partial}{\partial z} I$ by the change of the brightness of the images between adjacent planes in the focal stack. Optimal lateral resolution is obtained if the distance between adjacent planes corresponds to one Rayleigh unit $\sim \lambda / \mathrm{NA}$. If the sample has only small phase differences, it can be useful to use larger distances. Following Paganin and Nugent[7], we directly solve the TIE with the help of Fourier transformation. To avoid periodic artefacts, boundary pixels are padded internally. Since slowly varying parts of the phase become visible in intensity only after a longer propagation distance, we apply a small high pass filter to reduce low frequency artefacts.

Having obtained the phase image, we can also calculate a gradient phase image, which resembles the contrast of a DIC image. Therefore, we call it relief contrast.

For a comparison, see Figure 5 where brightfield (A), DIC (B), and relief (C) are shown together. The gradient of the phase can be calculated for any direction as it is possible to adjust the direction of the gradient in post-processing after the image has been acquired, which corresponds to a rotation of the Wollaston prism in traditional DIC.

The TIE was originally derived for a coherent setting in a paraxial approximation. Only with this boundary condition can the result be rigorously interpreted as a phase of an electromagnetic wave with quantitative meaning. However, the equation can describe paraxial intensity transport for partially coherent light in the context of Wigner distribution functions[8]. And even for incoherent light the TIE describes the conservation of energy for moments of non-paraxial light fields[9]. Therefore, we apply the TIE also for high numerical aperture and partially coherent illumination, which gives good contrast, but a direct quantitative interpretation of the phase would require further calibration, which is not currently available for ZEISS Axioscan 7.

A practical example is shown in Figure 6. Potato starch granules are shown in a section of potato tissue. While showing relatively low contrast in brightfield, the contrast is markedly enhanced both in the phase and the relief contrast version of the TIE results.

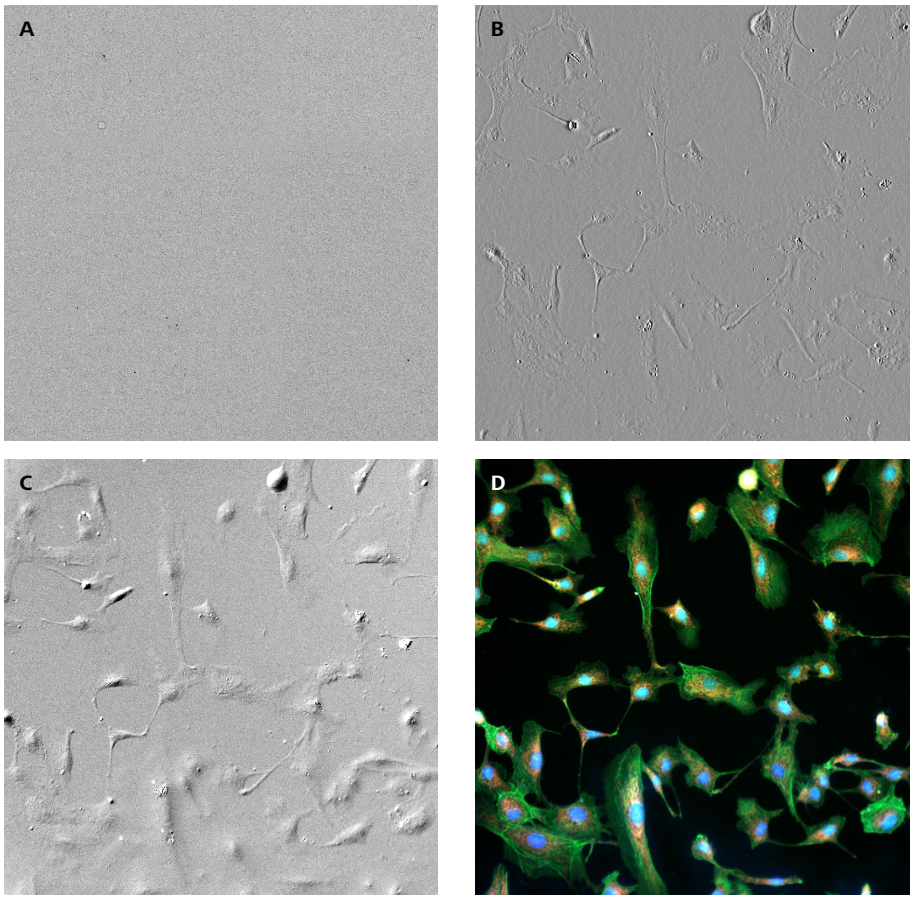

Figure 5 *FluoCellsTM No.1 (BPAE). A: Brightfield; B: DIC; C: TIE-relief; D: fluorescence.*

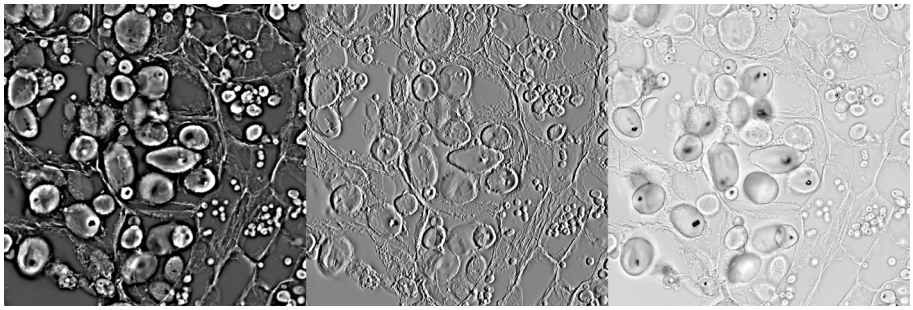

Figure 6 *Transmitted light images of potato starch. Left: TIE-Phase. Middle: TIE-Relief. Right: Brightfield.*

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