SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Pixel-by-pixel autofluorescence corrected FRET measurement and its implementation in the Pannoramic Confocal digital pathology scanner

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Members of the Examination Committee:	Péter Galajda, PhD
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The examination takes place at T. Bldg. 5th floor library, Faculty of Medicine, University of Debrecen, on February 29, 2024, at 11:00 am.

Head of the **Defense Committee**:	Árpád Tósaki, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on February 29, 2024, at 1:00 pm.

Introduction

Tumors are characterized by a high spatial heterogeneity. This makes it particularly difficult to determine the appropriate diagnosis and therapeutic approach. Even a single cell, different from the rest of the tumor, can influence the tumor's response to the applied treatment. Several methods are currently being developed to study tumor heterogeneity, such as spatial transcriptomics and spatial proteomics, but due to their complexity and cost, they are not used for diagnostics, only for exploratory clinicopathological research. Rapid advances in digital pathology not only allow the acquisition of tissue-level morphological images, but also the use of immunohistochemical markers to correlate these morphological features with the expression levels of one or two molecules. Nowadays, fluorescence pathology scanners are emerging and spreading, allowing the examination of several molecules simultaneously based on multiplex labeling. All this technological background has evolved from routine diagnostics, so it is intuitively evident that it can be applied to study the presence of markers of interest in large samples, even as rare events.

The Pannoramic Confocal developed by 3DHistech Ltd. is the first digital pathology scanner to provide confocal imaging in addition to traditional transmission and fluorescence imaging, allowing for spatial examination of thicker sections. This scanner was evaluated for confocality, stability, accuracy, linearity and sensitivity. Since interactions between certain molecules can often be a more sensitive indicator of the state of a cell than the expression level of those molecules, we also wanted to investigate whether the Pannoramic Confocal could be used to assess molecular interactions in tissue sections via Förster resonance energy transfer (FRET) measurements.

Digital pathology

In addition to tissue biomarker research, digital pathology is also becoming increasingly prominent in routine diagnostics. This is made possible by faster whole slide scanners and the decreasing cost of storage and computing power. Conventional transmission methods are currently used in routine diagnostic. Fluorescence imaging,

in particular confocal imaging, is an emerging area of automated whole slide scanning. Even in the 2021 edition of the European Society of Digital and Integrative Pathology's recommendation for best practice in digital pathology, fluorescence methods are only mentioned but not discussed. The introduction of confocal methods into automated slide scanners could improve their image quality and significantly increase z-directional resolution, enabling techniques that were previously not feasible with full slide scanners. Digital pathology and full slide scanning is an evolving field where new instruments and their continuous quality control are essential to ensure high standards.

Imaging system of the Pannoramic Confocal

The Pannoramic Confocal is a confocal fluorescence whole slide scanner, developed and manufactured by 3D Histech Ltd. (Budapest). Confocal imaging is provided by the Aurox cc88 aperture correlation confocal system. Its central component is a spinning optical disc with a grating pattern of transmissive and reflective areas. The light from the multispectral Lumencor LED light source is projected onto it via an optical filter system mounted in a motorized filter wheel.

The excitation light passes through the transparent elements of the grating pattern and through the objective lens illuminates the sample. The emitted light then passes back through the objective onto the spinning disc, where part of the light passes through and part of it is reflected. The optical system is designed so that the two fractions are imaged on the two halves of the same sCMOS camera sensor. The fluorescence excited by the patterned light passing through the grating and focused on the sample in the focal plane conjugate to the grating is transmitted through the transparent grating points to one half of the sensor. Since the transparent part is half of the total area of the plate, half of the photons from outside the conjugate foci are randomly transmitted through the grating to the same half of the sensor, while the other half are reflected from the reflecting parts of the plate and imaged by another optical path to the other half of the sensor. The transmitted and reflected images are used to compute the widefield and the confocal image as the sum and the difference of the two halves.

The microscope takes image frames of the selected area sequentially, with a few pixels overlap. From these frames, an image stitching algorithm creates a digital slide, which can be retrieved from computer memory at any time, and can be viewed using a suitable software as if navigating the slide with the microscope.

Point spread function and deconvolution

When a sample is examined with an optical microscope, the image that is formed is not a perfect representation of the sample. The spatial distribution of the image of a point light source is described by the point spread function (PSF) and the image becomes the convolution of the object and the PSF. For this reason, the distorting effect of the PSF cannot be eliminated by simple linear image processing methods, but only by deconvolution, for which several mathematical methods are available.

Molecular interactions in glioblastoma multiforme

Since glioblastoma multiforme (GBM) is highly resistant to both chemo- and radiotherapy, intensive research is focused on its resistance mechanisms. Among others, it has been found that the interaction between certain cell adhesion molecules and receptor tyrosine kinases may contribute to the development of chemo- and radioresistance. One such specific interaction occurs between EGFR and integrins. Integrins (e.g. the $\alpha 5\beta 1$ heterodimer) form multimeric clusters of different proteins in focal adhesion complexes that can generate signal transduction which prevents apoptosis. EGFR also occurs in these structures and can potentially initiate the same survival signaling. In addition, the interaction between EGFR and integrin $\beta 1$ (ITGB1) enhances cell detachment from the tumor, cellular motility and metastatic potential. In a previous publication, our group has shown that EGFR enhances ITGB1 expression and that higher expression of ITGB1 and EGFR decreases EGFR homoassociation and increases EGFR- ITGB1 heteroassociation, a phenomenon that contributes to the radioresistance of GBM. The heteroassociation of ITGB1 and EGFR is increased in higher grade astrocytomas and can therefore be used as a prognostic parameter, which appears to be more reliable than the expression level of

the interacting molecules. As similar potentially predictive interactions are present in many disease processes, molecular interactions are expected to play an increasingly important role in pathodiagnostics.

Förster resonance energy transfer (FRET)

For the measurement of molecular interactions, Förster resonance energy transfer (FRET) has been a popular tool for decades. Its strong distance dependence in the 1-10 nm range makes it suitable for the task, even in optical instruments that are otherwise limited to lower resolution due to diffraction.

The physics of FRET

FRET is a collision free, highly distance dependent photophysical process. During FRET, an excited fluorescent molecule (donor) transfers energy to an appropriate acceptor, which may have fluorescent properties. The energy transfer occurs via a long-range dipole-dipole coupling mechanism in a nonradiative manner (without photon generation). For FRET to occur, the donor and acceptor molecules must be spatially aligned, and the emission spectrum of the donor must overlap with the absorption spectrum of the acceptor. Since FRET efficiency (E) depends on the negative 6^{th} power of the distance, the phenomenon can be used as a spectroscopic ruler. FRET occurs only in the near field, which falls in the 1-10 nm range. When the distance between the donor and the acceptor is less than 1 nm, energy transfer between them is most likely to occur via collision, and when the distance reaches ~10 nm, E drops below 2% and photon emission from the donor dominates.

FRET measurement with microscopy

There are many methods for measuring FRET in microscopy, from single molecule measurements to approaches that describe the average distance of several molecules. Many of these do not require expensive instrumentation and/or do not degrade the sample, such as intensity ratio-based methods, which are best suited for the growing field of fluorescent protein-based biosensors with known donor/acceptor stoichiometry, and quantitative measurement methods that yield calibrated FRET

efficiencies independent of donor/acceptor stoichiometry. Among the latter, the spectral spillover-corrected (or three-cube) FRET method is a cost-effective and versatile approach that can be applied to flow cytometry as well as conventional fluorescence microscopy. In contrast to the also popular acceptor photobleaching technique, this technique can be used in combination with time-lapse and 3D image analysis.

Limitations of FRET measurement methods

The main limitation of all FRET measurement methods is the signal to noise ratio (SNR). The noise of all the fluorescence intensities measured is propagated into the noise of the FRET efficiency calculated from them. Assuming a Poissonian distribution of the detected photons with expected value λ, the SNR is $\sqrt{\lambda}$, so lower intensities are expected to increase the uncertainty of the calculated FRET efficiency to a larger extent. Samples with low SNR can be made suitable for FRET analysis by improving the quantum efficiency and photostability of fluorescent dyes, more efficient detection, optimization of FRET dye pairs, and improved mathematical and statistical approaches. Using the spectral spillover-corrected FRET method, it is generally possible to infer existing molecular interactions with confidence when FRET efficiencies of ~5% or more are seen.

FRET measurements and autofluorescence

The achievable SNR is also limited by the autofluorescence of the cells. Since cellular autofluorescence is typically less strong in the red region of visible light, it is preferable to use donor-acceptor dye pairs in this spectral region. To reduce the biasing effect of autofluorescence, its relatively stable spectrum can be used, so that its contribution to the optical channels of the FRET measurement can be estimated from the value in an independent reference channel. This method has previously been successfully introduced by our group for flow cytometric FRET measurements. Leavesley and his group have further developed the approach by introducing spectral unmixing and have shown that the method can be applied to study compartmentalized

signal transduction mechanisms. In another innovative approach, quantum dots were combined with fluorescent proteins for FRET measurements, using time-gated acquisition to exclude native fluorescence. However, the application of these two approaches requires an advanced technical background and special microscopes.

Aims

Literature data suggest that in many pathological processes, including tumor lesions, not only the number of molecules involved in signal transduction processes, but also the extent of molecular interactions between them can be an important diagnostic or prognostic/predictive marker.

FRET is a useful method to investigate the interaction between signaling partners, and its accuracy can be improved by optimizing the labeling and measurement protocols and by performing appropriate autofluorescence correction.

A major barrier to the diagnostic adaptation of FRET measurements is that some measurement procedures require specialized instrumentation and are typically slow for acquisition of large images. A solution to this problem may be to implement the appropriate FRET measurement procedure on an automated confocal fluorescence whole slide scanner using the fast expanding digital pathology toolbox.

Therefore, we have set the following objectives:

1. Investigate the imaging capability and suitability for quantitative measurements of the Pannoramic Confocal pathology scanner.

2. Develop an efficient autofluorescence correction procedure for intensity-based spectrally corrected microscopy FRET measurements that can be applied in a digital slide scanner.

3. Validate these autofluorescence corrected FRET measurements in digital pathology using conventional confocal microscopy.

Materials and methods

Multicellular 3D model grown on contact lenses

The contact lenses carrying the cells were fixed in an ascending series of acetone and washed with PBS. The sample was permeabilized and blocked with PBS containing 2% BSA and 0.1% Triton X-100 for 1 h and incubated for 90 min with the following primary antibodies: Alexa Fluor 647 anti-cytokeratin 19; guinea pig anti- cytokeratin 3 and Cy3 conjugated mouse anti-vimentin. After three washes with PBS/Triton X-100, samples were labeled with Alexa Fluor 488 conjugated anti-guinea pig antibody for 1 h and washed again three times. Nuclei were labelled with DAPI. Finally, the samples were fixed with 1% formaldehyde in PBS for 10 minutes, rinsed and mounted using Mowiol antifade.

Immunofluorescence labeling for FRET measurements

A172 glioblastoma and SK-BR-3 breast cancer cells were cultured in DMEM supplemented with 10% FBS at 37 °C under 5% CO_2 in a humidified incubator for 2 days on glass coverslips. The coverslips were washed with HEPES buffer supplemented with 4 mM glucose on ice, fixed with 1 % formaldehyde for 15 min, and washed three times. All subsequent steps were performed at room temperature. For direct labeling, cells were labeled with donor and acceptor labeled antibodies, both applied at saturating concentrations, in HEPES buffer containing 2% BSA for 30 min, with the following combinations: ab528-Alexa Fluor 546 + BIIG2-Alexa Fluor 647; ab528-Alexa Fluor 647 + BIIG2-Alexa Fluor 546; ab528-Alexa Fluor 546 + TS2-Alexa Fluor 647; ab528-Alexa Fluor 647 + TS2-Alexa Fluor 546. For positive controls of intramolecular FRET measurements, ab528-Alexa Fluor 546, BIIG2-Alexa Fluor 546 and TS2-Alexa Fluor 546 primary antibodies were used in the same way. Cells were washed again three times, followed by a similar labeling cycle with the Alexa Fluor 647 conjugated secondary antibody matching the isotype of the primary antibody. The final washing was followed by fixation and mounting in antifade.

Intramolecular FRET samples based on trastuzumab and pertuzumab monoclonal anti-HER2 antibodies were prepared as described in the direct labeling protocol above with the following antibody combinations: trastuzumab-Alexa Fluor 546 + pertuzumab-Alexa Fluor 647; pertuzumab-Alexa Fluor 546 + trastuzumab-Alexa Fluor 647. Both frozen N87 tumor sections and SK-BR-3 cells grown on glass surfaces were labeled in this manner.

Laser scanning confocal microscopy

All confocal microscope measurements were performed with an LSM 880 laser scanning confocal microscope (Carl Zeiss GmbH). The objective used was a 40× C-Apochromat water immersion (NA=1.2).

FRET measurements

For FRET measurements, the LSM 880 microscope was used in line scanning mode and all fluorescence emission channels were detected simultaneously by the instrument's 32-channel GaAsP detector. The confocal aperture was 1 Airy unit and the pixel size was set to 100 nm. The excitation in the autofluorescence channel was 488 nm and the detection range was 499-535 nm. The donor and the transfer channel were simultaneously excited with 543 nm laser light and detected in the 553-615 nm and 651-695 nm range, respectively. The acceptor channel was excited at 633 nm and detected in the range 651-695 nm.

Flow cytometry

Flow cytometric fluorescence and FRET measurements were performed with a NovoCyte RYB (Acea Biosciences/Agilent) cytometer. For autofluorescence measurements, the instrument's predefined FITC-H channel was used (488 nm excitation and 515-545 nm detection); the donor channel was PE-H (561 nm excitation and 576-596 nm detection); the transfer channel was PE-Cy5 mPlum-H (561 nm excitation and 650-670 nm detection); and the acceptor channel was APC-H (640 nm excitation and 650-670 nm detection). Evaluation, gating and correction factors were determined using FCS Express (De Novo Software) v7.

Pannoramic Confocal

Measuring the stability and relocation accuracy of the stage

TetraSpeck beads (d=500 nm) were imaged in the widefield imaging mode of the Pannoramic Confocal. To test stability, images were acquired every minute for 120 minutes from the same field of view. To measure the relocalization accuracy, 10-10 images were acquired by moving the stage one field of view after each image was captured and then moving it back into position. This sequence of operations was performed in both directions along both translational axes. In each case, the movement of 15 beads was tracked using the TrackMate Fiji plugin.

Measurement of illumination homogeneity

Illumination homogeneity was measured on blue fluorescence reference slides (Ted Pella). 20 images were recorded for each channel and averaged to reduce noise.

Determination of measurement linearity

The linearity of the instrument intensity response was measured using InSpeck green linearity calibration beads (ThermoFisher). The beads were measured in 15 focal planes with 1.2 μm step spacing, averaged intensity Z projections were performed, and after subtraction of the average background, the intensity of each bead was measured and averaged for each brightness category.

Determination of sensitivity

The sensitivity of the microscope was determined using QIFIKIT calibration beads, consisting of 5 bead populations with different known numbers of mouse IgG antibodies conjugated to the surface. The beads were labeled in a sorter tube with saturating concentration of Alexa Fluor 488 conjugated goat anti-mouse IgG in HEPES buffer, washed with 300 g, dried overnight on the slide surface and covered with Mowiol. The intensity of 1190 beads in widefield mode and 3196 beads in confocal mode was determined after background subtraction.

Determination of the point spread function and deconvolution

The point spread function (PSF) of the Pannoramic Confocal was determined using PS Speck beads. In each channel of the microscope, 100 slices were acquired with both 20× and 40× objectives at 200 nm Z-step spacing. PSF symmetry distribution map and theoretical PSF dimensions were generated using the PSFj Fiji plugin. Huygens software (SVI) was used to generate the empirical PSF from the measured images. Deconvolution was performed with the Huygens Classic Maximum Likelihood Estimation (CMLE) algorithm, limited to a maximum of 50 iterations.

Image analysis

Image analysis was done using the Fiji software. The plugin previously developed in our laboratory for calculating the traditional intensity-based spectrally corrected FRET measurement was rewritten to allow pixel-by-pixel autofluorescence correction and the fully automatic processing of larger image sets, as well as to be compatible with ImageJ 2.0. The new plugin is available via the FRET Imaging update page within Fiji.

Results

Testing the Pannoramic Confocal automated pathology scanner

The Pannoramic Confocal is an automated confocal pathology whole slide scanner that has the potential to enable rapid and automated investigation of molecular interactions. This requires that it maintains spatial stability during imaging, has a linear intensity response and is sensitive enough to detect proteins at low expression levels

Stability and relocation accuracy of the stage

A motorized stage with high stability and precision is an essential requirement for creating high-quality digital slides. To test the stability of the stage, fluorescent beads were recorded every minute for two hours. In the first ten minutes, slight movement along the X and Y axis was observed; the magnitude of the displacement was less than one pixel (<325 nm). The cessation of the movement could be explained by the onset of thermal equilibrium.

To determine the relocalization accuracy of the microscope, we moved the sample back and forth ten times by a single field of view and took images at the end of each cycle. Measurements were taken in all 4 main directions of the plane. In each case, the inaccuracy of the relocalization was less than the size of a pixel, with the magnitude of the error always being larger on the axis of displacement.

Based on our measurements, the Pannoramic Confocal sample handling is stable enough in both time and space to record good quality digital slides.

Homogeneity of illumination

For quantitative measurements and for good image stitching in digital slide generation, a sufficiently homogeneous intensity distribution of the illumination field is required. The illumination intensity distribution of Pannoramic Confocal in widefield mode was investigated on fluorescent slides with both 20× and 40×

objectives in the four fluorescence channels of the microscope. The center of illumination does not coincide with the center of the recorded image, which is due to the special optical system of the microscope. The homogeneity of illumination for both objectives is considered to be uneven and shows a slight wavelength dependence. It can be concluded that a correction procedure is necessary for proper operation. We have introduced a correction method, which performs intensity correction on all raw images using the image of a homogeneous fluorescence sample, acquired for each optical channel and objective. This method is suitable for the appropriate correction of images from small areas and spatially diverse samples and gives reliable results for quantitative measurements.

Linearity and sensitivity

In order to make quantitative measurements with a microscope, the intensity measured at a given pixel must be proportional to the number of photons produced there. This condition is particularly important when the ratio of the measured intensities is considered, as is typically the case for intensity-based FRET measurements. To be able to perform these measurements on biologically relevant samples, the microscope must be sufficiently sensitive at low expression levels. The linearity of the microscope intensity response was determined using InSpeck green fluorescence linearity calibration beads. The intensity response of the instrument was linear in both widefield and confocal modes, with R^2 of 0.9996 in widefield and 0.9997 in confocal imaging mode.

QIFIKIT beads were used to determine the sensitivity of the microscope in widefield and confocal mode. The beads consist of 5 subpopulations of different intensities, and the microscope was able to detect all 5 subpopulations in both imaging modes. The QIFIKIT beads have an average diameter of 10 μm, similar to an average cell, so we can assume that the 2000 epitopes/bead detected on QIFIKIT correspond to a similar order of magnitude of epitope density for fluorescently labelled cells.

Measurements on large area and thickness samples

The 3D imaging capabilities of the Pannoramic Confocal were tested on a cell multilayer grown on the surface of a contact lens for corneal limbal stem cell transplantation. The sample is composed of a mesenchymal-like "feeder" cell layer that is vimentin-positive, and cytokeratin 19 and cytokeratin 3-positive epithelial cells that differentiate in different directions. These three differentiation markers were labeled by immunofluorescence and nuclear DNA was visualized by DAPI staining. A quarter contact lens was imaged in confocal mode with the Pannoramic Confocal over the full thickness of the sample, which in this case was 50 μm due to the curvature of the contact lens. A small area of the sample was also imaged with an LSM 880 microscope. In these images, we examined the intensities measured in cell-free and high intensity cellular areas. They show that the Pannoramic Confocal has better contrast than the LSM 880 microscope in two channels and the same contrast in the other two channels. A clear advantage of the Pannoramic Confocal is that we were able to achieve comparable image quality with a voxel time of 1 μs compared to the 10 μs voxel time of the LSM 880.

Confocal performance and deconvolution

The measured point spread function (PSF) values of the Pannoramic Confocal are significantly higher in all directions than the theoretical values for a laser scanning confocal microscope, and the symmetry is also significantly below the optimal value of 1. For this reason, it is not possible to run deconvolution algorithms on images acquired with the Pannoramic Confocal using a theoretical PSF model. Although the PSF is not symmetric, its spatial distribution can be considered homogeneous, which allows deconvolution using an empirically determined system PSF for each channel. The empirical PSF created from images of sub-resolution sized beads with the Huygens program was suitable for deconvolution. The deconvolution of surface-stained calibration beads resulted in a one-pixel-wide ring in all the three studied fluorescence channels. Based on this, joint development with the microscope

manufacturer now allows on-the-fly deconvolution during acquisition in the Pannoramic Confocal.

Pixel-by-pixel autofluorescence corrected FRET

The spectral spillover corrected FRET method is a cost-effective and versatile measurement technique that can be used in flow cytometry and various types of fluorescence microscopy, but its accuracy may be reduced by sample autofluorescence. To overcome this, we implemented and tested a pixel-by-pixel autofluorescence correction method.

Pixel-by-pixel autofluorescence correction algorithm

To calculate the pixel-by-pixel corrected FRET efficiency, we need four fluorescence channels: In I_0 we measure the intensity of autofluorescence; I_1 is the donor channel; I_2 is the transfer channel; in which we detect the emission of the acceptor under excitation corresponding to the donor dye; and I_3 is the acceptor channel.
FRET efficiency (E) can be calculated from these four intensities measured on samples double labeled with donor and acceptor according to the following equation:

$$E = \frac{A}{\alpha \cdot (epsR-1) \cdot [B_2 I_0 S_4 + B_1 I_2 S_6 + I_1 (S_2 - B_2 S_6) - B_1 I_0 S_2 - I_2 S_4] + A}$$

Where

$$A = S_2\{B_2 I_0 + I_1 S_1 + I_3 S_2 - S_2 (B_3 I_0 + I_1 S_3) + B_1 I_0 (S_2 S_3 - S_1) + (B_3 I_0 - I_3) S_1 S_4 +$$

$$(B_3 I_1 - B_1 I_3)(S_2 S_5 - S_1 S_6) + I_2 [B_1 S_5 - B_3 S_4 S_5 + B_3 S_6 + S_3 (S_4 - B_1 S_6) - 1] -$$

$$B_2 [I_0 S_3 S_4 + I_3 (S_6 - S_4 S_5) + I_1 (S_5 - S_3 S_6)]\}$$

The *epsR* is the product of the molar absorption coefficients of the donor and acceptor dyes at each other's excitation wavelength divided by the molar absorption coefficients at their own excitation wavelengths.

S_1, S_3 and S_5 are the donor dye specific spillover factors measured on a donor-only sample, while S_2, S_4 and S_6 are the acceptor specific spillover factors measured on an acceptor-only sample. B_1, B_2 and B_3 measure the spillover of autofluorescence and are determined on unlabeled cellular samples.

The parameter α corrects for the difference in quantum efficiency between the acceptor and donor dyes and their detection efficiency. To determine this parameter, a sample containing only donor and only acceptor dyes is required.

As these factors are determined on labelled cellular samples in the conventional measurement method, an unknown contribution of cellular autofluorescence to the signal will introduce random error. To avoid this, we developed cell-free, long-lasting calibration standard slides, which can also be used for the illumination homogeneity correction of the Pannoramic Confocal.

If no autofluorescence correction is applied, only the background of the instrument is subtracted from each channel (non-corrected evaluation). It is also possible to determine the average autofluorescence intensity in channels I_1, I_2 and I_3 on unlabeled samples and then subtract these values from all three channels as constants; this method is called average autofluorescence correction.

Autofluorescence correction on unlabelled samples

To determine how efficient the pixel-by-pixel autofluorescence correction is compared to the average autofluorescence correction, the two methods were first tested on unlabeled A172 and SK-BR-3 cells. The efficiency of the correction methods was evaluated in all three spectrally corrected channels used for intensity-based FRET measurements. For unlabeled samples, we expect that with perfect autofluorescence correction, the average intensity of the resulting images will assume the value of 0 and the per-pixel variance will be low. The cell-by-cell integrated autofluorescence intensities were close to 0 for both methods in the three fluorescence channels tested (donor, transfer, acceptor), i.e., when averaged, both methods remove cellular background fluorescence. However, with average autofluorescence correction, the averages have a larger range around the 0 value, since the spatially

fluctuating values are corrected by an average constant. To support this, we determined the ratio of the SD of images obtained with pixel-by-pixel and average autofluorescence correction, which shows that for A172, pixel-by-pixel correction reduces the SD by 50-60%, while for SK-BR-3 cells by 20-40%. These results suggest that the pixel-by-pixel correction reduces the average autofluorescence intensity to zero, as expected, and reduces the per-cell and per-pixel SD formidably. Of course, the Poissonian noise of the detection still burdens the values measured in each pixel, so a fully uniform corrected intensity cannot be expected. Nevertheless, derived parameters such as FRET efficiency will be more accurate with pixel-by-pixel autofluorescence correction than with average correction.

FRET measurements between pairs of biologically relevant molecules

We have measured FRET efficiencies between EGFR and ITGA5 and ITGB1 on A172 cells. Since these integrins are present in 5-fold and 8-fold higher amounts on the A172 cell surface compared to EGFR, FRET efficiency between epitopes was measured in two ways for both EGFR-integrin pairs: in one sample the donor-labelled antibody was bound to EGFR, while in the other sample it was bound to the integrin. This procedure eliminates the possible biasing effect of the acceptor/donor ratio, which could increase the FRET E value due to random association with a large number of acceptors. The evaluation was performed without autofluorescence correction and with both average and pixel-by-pixel autofluorescence correction. In these samples, FRET efficiencies followed the acceptor/donor ratios well, i.e., in cases where EGFR was present as an acceptor, lower FRET efficiencies were measured than when it was present as a donor. The relationship between the values obtained without correction and with pixel-by-pixel correction is coherent between cases, however, the FRET efficiency obtained with average correction in the ITGA5→EGFR and ITGB1→EGFR samples is significantly lower compared to the other two methods, less than -0.1 in the former case. This indicates that the average autofluorescence correction can produce strong negative artefacts, especially in samples with low FRET efficiency.

When ITGB1 was the acceptor, the average autofluorescence correction resulted in a very high FRET efficiency, and 0 when ITGB1 was the donor. For the same samples, the pixel-by-pixel correction resulted in FRET efficiencies higher than zero in both measurement directions, with ratios that followed well the ratio of donor to acceptor dyes. This confirms that the average autofluorescence correction performs worse than the pixel-by-pixel autofluorescence correction. It can also be observed that for all biologically relevant samples, the pixel-by-pixel autofluorescence correction falls between the results measured by the other two correction methods. The signal/autofluorescence ratio of the sample determined whether the average corrected or the non-corrected FRET E took the highest or the lowest value. In those samples where the signal/autofluorescence ratio was higher in the donor channel, the noncorrected FRET values were high, while in those samples where the signal/autofluorescence was high in the acceptor channel, the average autofluorescence correction gave high FRET efficiencies.

FRET measurements on positive controls

Positive controls were created by labeling with acceptor-conjugated secondary antibodies the donor-conjugated primary antibodies bound to low and high expression epitopes. This approach yields the same acceptor/donor ratio regardless of expression, so that potential differences in FRET efficiencies are not caused by changes in dye ratios. The average autofluorescence corrected FRET efficiencies followed the trend in signal/autofluorescence ratios, suggesting a tendency for the average correction to bias the results. No such relationship was found for FRET efficiencies obtained without correction and with pixel-by-pixel autofluorescence correction. However, FRET efficiencies with pixel-by-pixel correction were very similar for the two mouse monoclonal antibodies (anti-EGFR and anti-ITGB1) but differed from those measured on rat monoclonal anti-ITGA5. Assuming that the complexes of donor-labelled primary and acceptor-labelled secondary antibodies are more similar when using the same mixture of secondary antibodies, the difference between FRET complexes containing rat and mouse antibody is acceptable. However, the nearly identical FRET

E values measured for the mouse-anti-mouse immunocomplexes at low and high specific signals in the presence of the same autofluorescence clearly indicate that the pixel-by-pixel autofluorescence correction is better than the average correction or than using no correction at all.

For further analysis, pixels from EGFR and ITGB1-based positive control samples were pooled and then divided into three groups according to autofluorescence intensity. The FRET values calculated without autofluorescence correction are inversely proportional to autofluorescence; at low autofluorescence the FRET efficiency overlaps with the pixel-by-pixel corrected FRET efficiency, but as autofluorescence increases, the measurement becomes more under-corrected. With average autofluorescence correction, the global average is far apart for EGFR and ITGB1. In this case, the FRET efficiencies of pixels with high autofluorescence overlap with the FRET efficiencies measured with pixel-by-pixel correction, and pixels with low autofluorescence are overcorrected. The FRET efficiency measured with pixel-by-pixel autofluorescence correction was independent of the magnitude of autofluorescence.

From the results presented, it was concluded that the pixel-by-pixel autofluorescence correction increases the accuracy of the FRET calculation, and eliminates the bias caused by no correction or average correction at low or high signal/autofluorescence ratios.

FRET measurement with the Pannoramic Confocal automated pathology scanner

Based on the fact that the optical properties of the Pannoramic Confocal automated pathology scanner make it suitable for quantitative measurements, we implemented the pixel-by-pixel autofluorescence correction FRET measurement in the scanner.

On N87 cell line-derived frozen sections of tumor samples grown in mice, the HER2 oncoprotein was labeled with two monoclonal antibodies conjugated to donor and acceptor dye, respectively, binding to epitopes in close proximity to each other. The sample was then measured using both the Pannoramic Confocal and an LSM 880

confocal microscope, and FRET efficiency maps were generated using pixel-by-pixel autofluorescence correction. The median FRET E measured with the two instruments was nearly the same, with a narrower FRET distribution measured with the Pannoramic Confocal at similar acquisition speeds.

The FRET efficiencies measured on the SK-BR-3 cell monolayer grown on coverslips were nearly identical to those measured on the frozen sections after the same labeling protocol. Also, in this case, no significant difference was found between the FRET efficiencies measured with the two microscopes. We conclude that the Pannoramic Confocal is suitable for accurate FRET measurements on both adherent cell cultures and cryosectioned tissues. Consequently, our acquisition and image processing algorithms have been incorporated into the scanner software.

Discussion

The Pannoramic Confocal is an automated confocal whole slide scanner that has the potential to enable rapid and automated investigation of molecular interactions. To determine this, we investigated the main imaging parameters of the Pannnoramic Confocal. Where relevant, we compared the Pannoramic Confocal with an LSM 880 laser scanning confocal microscope.

The Pannoramic Confocal has motorized sample handling. The temporal stability and the relocalization accuracy of the instrument was investigated. The microscope was found to be temporally stable, with a sample displacement of less than one pixel over a two-hour time interval; the relocalization inaccuracy did not reach one pixel after 10 repetitions. As the object stage inaccuracies are of sub-pixel magnitude, this does not affect in practice the quality of measurements with the Pannoramic Confocal.

To perform quantitative measurements and to ensure good image fusion during the creation of a digital slide, a homogeneous intensity distribution of illuminating light is required. When examining the illumination intensity distribution of the Pannoramic Confocal, we found that the center of illumination in all channels of the microscope was off-center, due to the special optical system of the Aurox cc88 confocal unit. The illumination in all channels was inhomogeneous with a slight wavelength dependence, making it essential to use a proper correction procedure, especially for quantitative measurements. As a result of this observation, a new correction method is now available that performs intensity correction before the confocal image is calculated, based on a homogeneous fluorescence sample with images acquired separately for each optical path. This method is suitable for the correction of small images and also gives more accurate results for quantitative measurements than the standard post-acquisition statistics-based correction.

A critical property of any microscope is the ratio of the measured intensity to the number of photons from a given pixel. Ideally, the intensity response of a microscope is linear. A linear intensity response is particularly important when comparing the

ratio of intensities measured in two spectrally different fluorescence channels, which takes place for example in the case of FRET measurements. Since the intensity response of the microscope is linear in all imaging modes, it can be assumed that the microscope is suitable for quantitative measurements. After performing the linearity measurements, we examined the sensitivity of the microscope. Adequate sensitivity is of paramount importance for quantitative measurements on biological samples with normal expression. Using the Pannoramic Confocal, ~2000 epitopes/cell can already be reliably quantified.

By investigating the imaging quality of the Pannoramic Confocal, we found that even for challenging samples such as a quarter contact lens mounted on a slide, the microscope can generate a digital slide without visible misalignment, despite the complex 3D structure and large area of the sample. Comparing the contrast ratios of the Pannoramic Confocal and the control LSM 880 microscope on this sample, we found that the Pannoramic Confocal captured images with better contrast ratios in two channels and the same contrast ratio in the other two tested channels. It is also worth noting that this was achieved in one tenth as long voxel time as with the control microscope. The fast imaging of the microscope can be extremely advantageous for large samples but can also be used to achieve longer exposures and better signal-to-noise ratios when needed.

In addition, confocal image quality can be further improved by deconvolution using the spatial homogeneity of the point spread function and the fact that, although a theoretical PSF cannot be modelled, with an appropriate calibration sample an empirical system PSF functions can be generated for each optical channel.

Overall, images created by the Pannoramic Confocal, with appropriate correction for illumination inhomogeneity, are suitable for performing quantitative measurements, including spectral spillover corrected FRET measurements.

Our choice of spectrally corrected (three-cube) FRET is a cost-effective and versatile measurement technique for molecular interactions, which, in addition to flow

cytometry, can be used in different modes of fluorescence microscopy. However, the results of such measurements can be affected by the autofluorescence of the sample. Tissues are often characterized by high and spatially variable autofluorescence, therefore, before implementing spectrally corrected FRET measurements on the Pannoramic Confocal, we tested possible solutions to eliminate the confounding effect of autofluorescence using a confocal laser scanning microscope.

Average autofluorescence correction is a simple approach, which can be easily used for almost all intensity-based FRET measurements, but, because it corrects the spatially heterogeneous signal by a constant, it has a potential for error. For this reason, we have adapted and implemented a procedure previously used in flow cytometry that can correct for the biasing effect of autofluorescence on a pixel-by-pixel basis in microscopic measurements. We have also implemented the new evaluation algorithm for the measurement protocol in our previously developed Fiji (ImageJ) RiFRET plugin, and added the possibility of fully automatic evaluation of large image sets.

The correction methods were first tested on unlabelled cells. On such samples, perfect autofluorescence correction theoretically results in low SD images with zero mean intensity. With average autofluorescence correction, the mean intensity of the corrected images was close to 0, while the SD was equal to the SD of the noncorrected images. In contrast, with the pixel-by-pixel autofluorescence correction procedure, not only did the mean intensities fall close to zero, but the standard deviation of the pixel values was also significantly reduced.

To compare the two methods, FRET measurements were performed on biologically relevant samples and their corresponding high FRET efficiency positive controls. For the ITGA5 →EGFR sample, the average autofluorescence correction resulted in a strong negative artefact. Since here the autofluorescence is high, and the acceptor signal and the FRET efficiency are low, this provides further evidence that the average autofluorescence correction procedure should be avoided for samples with low

signal/autofluorescence ratios, and instead the use of a pixel-by-pixel autofluorescence correction procedure is recommended. In contrast, for uniform and low autofluorescence samples with high labeling intensity, the average autofluorescence correction is also expected to give the correct result.

To further explore the differences between the correction methods, low and high autofluorescence regions were identified in the EGFR-based positive control sample, and FRET efficiencies were determined without, with average, and with pixel-by-pixel autofluorescence correction. The distribution of the resulting FRET efficiencies was examined over the whole image, as well as in the low and high autofluorescence regions. With the pixel-by-pixel autofluorescence correction, FRET histograms with a similar normal distribution were obtained in all three cases. Without autofluorescence correction, the distribution of the high autofluorescence area was distorted, while with the average autofluorescence correction, a similar distortion was observed in the low autofluorescence area due to overcorrection.

To investigate the background of these biases, all pixels of EGFR and ITGB1-based positive control samples were pooled and divided into low, medium and high groups according to autofluorescence. Examining these results, we found that without correction and with average autofluorescence correction, FRET efficiency is not independent of the autofluorescence, but with pixel-by-pixel autofluorescence correction it is independent of the magnitude of autofluorescence.

From these results, we conclude that pixel-by-pixel autofluorescence correction increases the accuracy of the FRET calculation and eliminates the bias observed in samples with low signal/autofluorescence ratios.

The pixel-by-pixel autofluorescence correction method implemented in the Pannoramic Confocal was tested on trastuzumab/pertuzumab intramolecular FRET samples by performing this labeling on a SK-BR-3 cell line grown on a glass surface and on frozen sections from N87 cell line derived tumor grown in mice. A Zeiss LSM 880 laser scanning microscope was used as a control. We found no difference in FRET

efficiencies between the two microscopes, concluding that the Pannoramic Confocal is suitable for accurate FRET measurements of both cell cultures and tissue sections. In addition, the Pannoramic Confocal is capable of acquiring images with similar or better contrast ratios than the control LSM 880 at lower exposure times and is suitable for scanning whole slides. Overall, the implementation of pixel-by-pixel autofluorescence corrected FRET measurements in the Pannoramic Confocal provides the technical basis for extending the measurement of molecular interactions from research to the field of pathodiagnostics. Based on these results, clinical samples are currently being collected to further investigate EGFR-integrin interactions.

In addition, several studies have recently been published that have identified additional pairs of potentially diagnostically relevant molecules. For example, the extent of HER2-HER3 heteroassociation is highly predictive of metastasis and recurrence in breast tumors, and the importance of the same interaction in colorectal carcinomas has been raised. HER2-HER2 homoassociation has also been shown to be a good prognostic marker in early breast tumors. The interaction of PD-1 and PD-L1 in melanoma has been shown to be independent of the amount of PD-L1. High interaction in non-small cell lung cancer and melanoma worsens survival prospects, but also increases the therapeutic efficacy of PD-1 and PD-L1 inhibitors. All these predictive interactions can potentially be investigated with high efficiency on whole sections or section microarrays using the improved Pannoramic Confocal.

Summary

The rapid development of digital pathology allows morphological imaging at the tissue level. Using immunohistochemical markers, these morphological features can be correlated with the expression level of one or two molecules in the same slide. The recent emergence of fluorescence pathology scanners, however, allows multiplex detection of several molecules in the same sample. The Pannoramic Confocal is the first digital pathology scanner to provide confocal imaging in addition to conventional transmission and fluorescence imaging. We evaluated the scanner for confocal image quality, stability, accuracy, linearity and sensitivity. The point spread function of the confocal image was asymmetric, but spatially invariant; therefore, we have implemented a deconvolution algorithm for the instrument. The stability and relocalization inaccuracy in the X-Y dimensions were well below the resolution limit. The intensity response was linear ($R^2 \geq 0.9996$). Calibrated measurements showed that $\geq$ 2000 molecules per cell could be reliably detected and mapped using indirect labeling. We found that the microscope illumination is asymmetric and inhomogeneous. To overcome this, we introduced a correction method that is suitable for the proper correction of images also from small areas as well as spatially diverse samples and gives reliable results for quantitative measurements. Based on this and findings on instrument precision, the Pannoramic Confocal appears suitable for measuring molecular interactions in histological sections.

Förster resonance energy transfer (FRET) is a popular tool for studying molecular interactions due to its high distance sensitivity in the 1-10 nm range. The intensity-based, spectrally corrected FRET measurement method has the advantage of being suitable for three-dimensional as well as time-lapse measurements. To use this method in microscopy with pixel-by-pixel autofluorescence correction, the flow cytometric cell-by-cell autofluorescence correction algorithm has been adapted. The implementation of pixelwise autofluorescence correction improves the accuracy of measurements and is particularly recommended for samples with low signal/autofluorescence ratios and spatially variable autofluorescence intensities. The Fiji/ImageJ plugin written to perform the analysis is freely available.

The measurement procedure was also tested with the Pannoramic Confocal, where FRET efficiencies measured on cells and tissue cryosections were in accordance with those measured as control with an LSM 880 laser scanning microscope. Based on this, the pixel-by-pixel autofluorescence corrected FRET measurements have been implemented in the Pannoramic Confocal digital pathology scanner software, providing the methodological and instrumental basis for diagnostic FRET measurements.

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Candidate: István Rebenku
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Rebenku, I.**, Lloyd, C. B., Szöllősi, J., Vereb, G.: Pixel-by-pixel autofluorescence corrected FRET in fluorescence microscopy improves accuracy for samples with spatially varied autofluorescence to signal ratio.
   Sci. Rep. 13 (1), 1-15, 2023.
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3. Bankó, C., Nagy, Z. L., Nagy, M., Szemán-Nagy, G., **Rebenku, I.**, Imre, L., Tiba, A., Hajdu, A., Szöllősi, J., Kéki, S., Bacsó, Z.: Isocyanide Substitution in Acridine Orange Shifts DNA Damage-Mediated Phototoxicity to Permeabilization of the Lysosomal Membrane in Cancer Cells.
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