Poster Session

Presented in the
Exhibitor Ballroom
Wyndham Grand Pittsburgh Hotel

Tuesday, May 23, 2017

10:20-11:20 am
And
3:35-4:00 pm

Listed in alphabetical order by First Author

Evaluation of an Automated Tissue Sectioning Machine for Digital Pathology

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Content
Automation and digital pathology are the trends for future anatomic pathology with the increasing workload in histology laboratories. While tissue processing, embedding, staining and coverslipping, and digitizing have been available for automated use, tissue sectioning appears to be the biggest roadblock to a fully automated histology process. In this study we were aimed to investigate a tissue automated sectioning machine for both clinical and research use.

Technology
Tissue auto-sectioning machine AS-410 (Dainippon Seiki Co. LTD., Japan) which has the abilities of tissue detection, barcode reading and printing, and 3-8 µm tissue preparation, was used by this study. Nanozoomer 2.0HT (Hamamatsu, Japan) scanner was used to acquire the Whole slide images (WSI) of the H&E stained slides at a resolution of 0.46 µm/pixel.

Design
Totally 77 surgical resection blocks of various organs embedded with standard paraffin were sectioned automatically using AS-410 at 5 µm with the default setting (Setting A). 10 slides per block were sectioned and the last 5 slides were stained with H&E, digitized with WSI scanner, and evaluated by image scientist and pathologist. The image scientist scored the images base on the extent of imperfection (Evaluation I), while the pathologist scored the images based on the clinical diagnosis purpose (Evaluation II). Both scoring systems were scored from 1 to 5, with 1 the worst quality and 5 the highest quality. Tissues with unsatisfied score were sectioned with modified setting (Setting B), and evaluated again by the same image scientist and pathologist with the same scoring systems. And the scores from the two different settings were compared. Auto-trimming and barcode reading and printing of AS-410 were also evaluated.

Results
The AS-410 provided auto-trimming function to detect exposed tissue for cutting, accomplished by the installed camera and calculation software. It read sample information and printed barcode as well as input text and automatically generated slide order information. It produced good quality of sections for most cases with median score more than 4 in both Evaluation I and Evaluation II using setting A **(Figure 1a&b).** The scores of the unsatisfied blocks sectioned with setting A improved significantly when sectioned with setting B **(Figure 1c&d).**

Conclusion
The AS-410 tissue sectioning machine produces high-quality sections with clinical standard paraffin tissue blocks of a variety of organs with proper settings. It promises high automation with sound sectioning quality in the era of digital pathology for both clinical and research use.

Notes: The affiliation of Xiujun Fu and Yukako Yagi was Massachusetts General Hospital when this research was performed.

Figure 1 on next page

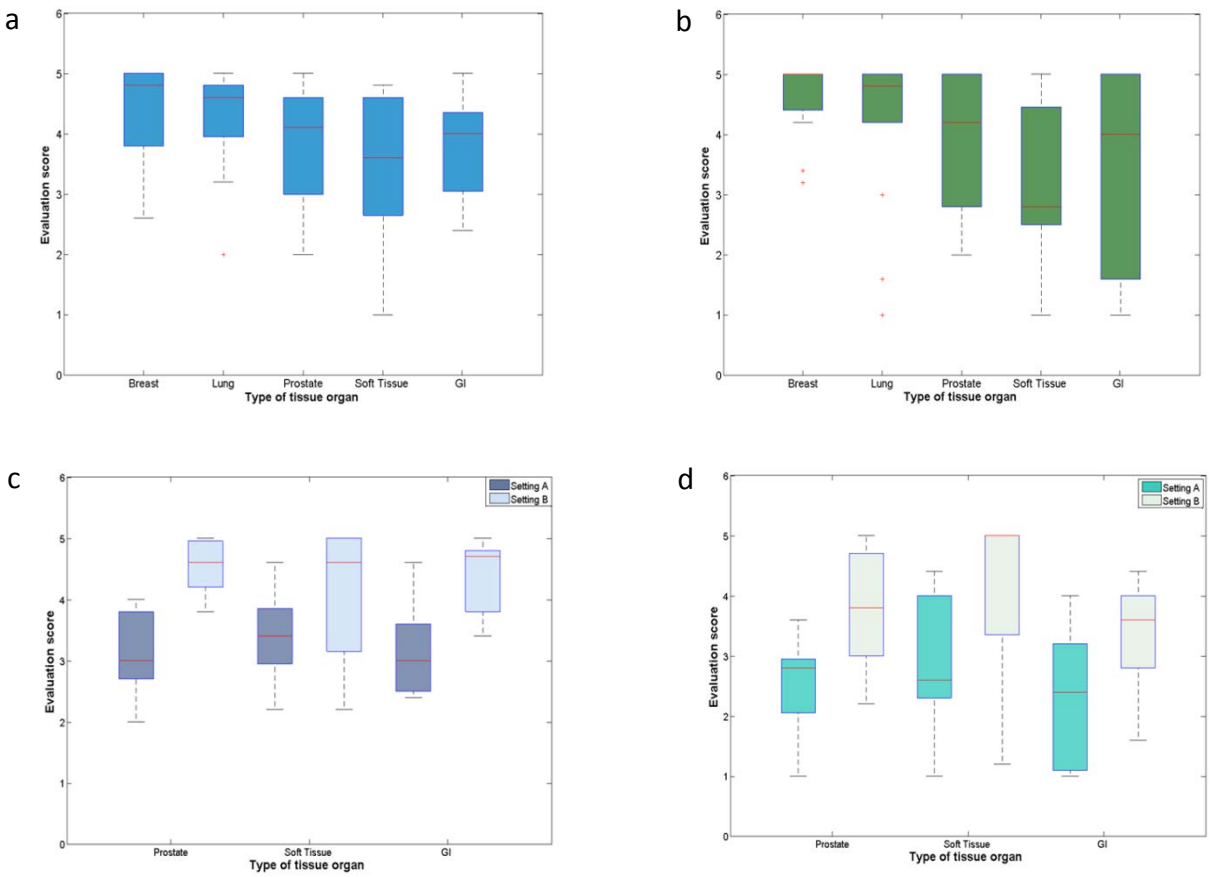

Figure 1 Evaluation scores given by imaging scientist (a, c) and pathologist (b, d).

Color Image Segmentation Using Multi-Level Thresholding: Applications for Computer-Automated Ki-67/SOX-10 Indexing

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University of Pittsburgh School of Medicine[1], University of Pittsburgh Department of Pathology[2], University of Pittsburgh Department of Dermatology[3]

Content
The Ki-67 labelling index has been shown to be a valuable prognostic indicator in various carcinomas including brain, breast, and skin tumors. However, adaptation into daily practice is met with challenges related to mode of assessment. “Eye-balling” is the least expensive and most widely used method but has poor reliability and reproducibility. Automated counting by image analyzers is the current gold-standard but has been shown to be expensive and impractical. In this study, we developed a custom computer-based algorithm to automatically calculate Ki-67 index in 3 Ki-67/SOX-10 dual-stained melanoma images with an emphasis on color-based image thresholding.

Technology
An Omnyx VL120 Scanner was used for digital slide scanning.
ImageJ Windows 64-bit was used for manual cell counting.
The algorithm was developed and implemented using Matlab 2016b.

Design
Ki-67/SOX-10 dual-stained slides high-grade melanomas were chosen for this study. Ki-67 is represented by red and SOX-10 by brown nuclear staining in this assay. Images were digitally uploaded using an Omnyx VL120 Scanner and magnified to 40x. Screenshots measuring approximately 1100x1700 pixels were downloaded for analysis. Cells were manually counted using ImageJ Win-64. The images were uploaded into Matlab 2016b for further automated Ki-67 indexing. The algorithm began with image upload and conversion from RGB to HSV color space. Multi-level thresholding was performed on the hue histogram to separate red/brown dual-stained (Ki-67/SOX-10 stained melanocytes) cells from brown only (SOX-10 stained melanocytes). Blob-analysis was then performed for noise reduction and a Ki-67 index was generated from the processed images. Indices calculated with the algorithm were compared to manual counts using a paired t-test.

Results
For n=3 images we found no significant difference between the computer-automated index and a manual count ($p = 0.06$).

Conclusions
Many image thresholding algorithms are based on image pixel intensity, relying on the continuity of gray-scale values for thresholds. These methods are not applicable for colored images in RGB format however, conversion to HSV places color values on a contiguous axis suitable for thresholding. Color thresholding can be a powerful tool in any application requiring quantification of colored bioassays.

Proposed Criteria for Rapid On-Site Evaluation (ROSE) Telecytology Validation: The UPMC Experience

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Content
Telecytology is being increasingly used at many centers. The most common mode of telecytology employed for remote rapid on-site evaluation (ROSE) is real-time streaming. However, most guidelines for digital pathology validation were developed for whole slide imaging. Therefore, the aim of this study was to establish practical methods for telecytology validation using real-time live video microscopy.

Technology
An Olympus DP71 camera attached to an Olympus BX51 microscope was used to stream 640x480 images over a 1 MB secure network. CellSens Standard 1.11 software was accessed via Citrix to remotely view streamed images.

Design
Four cytopathologists prospectively and collectively evaluated 60 CT-guided fine needle aspiration ROSE cases first using glass slides and immediately thereafter by another cytopathologist via telecytology. Glass slide and remote digital ROSE interpretations were compared to each other and final cytology diagnoses. Difficulties experienced during the study period were recorded.

Results
ROSE matched the final diagnoses in 98% of cases for both telecytology and glass slide microscopy (Table 1). The single discrepant case called “negative for malignant cells” at ROSE with both glass slides and telecytology revealed malignant cells only in the cell block (sampling issue). However, there was 100% concordance for ROSE diagnoses rendered digitally vs. by glass slide. Technical difficulties were identified in 17 cases (28%), with the majority (82%) occurring in the first half of the study.

Conclusion
Telecytology using live video microscopy can be validated prospectively during routine clinical ROSE service by showing non-inferiority of diagnostic error rates and concordance rates between digital and glass modalities. Prospective collection of cases helps avoid sample bias and is easy to perform, but may take longer to complete than a retrospective study. Validation helped “stress test” the telecytology system to resolve technical issues without compromising user performance.

Table 1. Digital vs Glass slide comparisons

ROSE comparison	Concordance (n=60)
Digital vs Glass slide on-site interpretation	60 (100%)
Digital vs Glass slide final diagnosis	59 (98%)

Standardized Playscripts for Digital Pathology Vendor Demonstrations

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Content
There are many vendor solutions that may need to be evaluated for an institution interested in adopting digital pathology. In order to find the right fit, demonstrating that a solution can handle digital workflow processes is imperative for the vendor evaluation process. Although some pathology workflows are routine, each institution may have unique workflow requirements. Crafting playscripts that describe typical workflow scenarios has previously proven to be useful to assess laboratory information system vendor performance. Therefore, the aim of this project was to develop a standard set of playscripts that could be used to appraise a digital pathology system.

Technology
Digital pathology solutions that offered hardware (scanners) and/or software (viewing and sharing of digital slides) for primary diagnosis were evaluated.

Design
Our institution invited several digital pathology vendors to demonstrate their solution to our informatics team. Prior to their visit, we documented our workflow requirements for routine surgical pathology practice. As part of our evaluation process, vendors were asked to demonstrate how their system would deal with these specific institutional workflows. Scorecards were used by stakeholders to evaluate vendor performance with these given playscripts.

Results
Five playscripts were created [Table 1]. Key areas of focus included laboratory system integration, viewing single and multi-part cases, review of prior patient digital pathology slides, viewing multiple imaging modalities (e.g. gross images and digital slides), intra- and inter-departmental consultation, and image analysis.

Conclusion
Employing standard playscripts for a digital pathology workflow proved to be extremely helpful during vendor demonstrations to evaluate their system's ability to be potentially implemented at our institution for primary diagnosis. We also recommend using such playscripts to objectively compare different digital pathology systems.

Table 1. Digital Pathology Playscripts

Scenario	Workflow Description
#1	We receive a 3-part biopsy case from a screening colonoscopy. Initial H&E's are cut and a complete diagnosis can be made from these slides.
#2	We receive a biopsy from a colonic mass in a patient with a prior history of malignancy. An initial H&E recut is made, but additional levels and some immunostains are required plus prior history needs to be reviewed. An intradepartmental consultation is needed for this case.
#3	We receive a surgical resection of a pancreatic mass that was previously biopsied by fine needle aspiration and core biopsy at our institution. Prior to gross examination, an intraoperative consultation was performed. Comparison with prior material is necessary. Gross images of the specimen were obtained and biomarker immunostains need to be ordered. This case is also selected to be presented for tumor board.
#4	We receive a consultation request from an outside institution to be performed on digital images from a foreign institution.
#5	We receive a case with invasive ductal carcinoma of the breast. The tissue is stained with estrogen receptor, progesterone receptor, Her2 and Ki-67. Image analysis is required and needs to be reported in the final diagnosis.

Recorded Pathology Didactic Lectures for Global Online Distribution: A 12-Year Experience

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Content

Pathology didactic lectures are typically available at academic teaching centers, professional society meetings where participants pay for registration, or via organizations that offer paying members access to live or archived webinars and podcasts. However, there are limited free online resources to didactic lectures. As the University of Pittsburgh Medical Center (UPMC) pathology department expanded into a multi-hospital healthcare system spread over hundreds of miles, there was a need to provide remote continuing education to its department members. For this purpose, didactic lectures were shared online not just to UPMC staff, but also globally. The aim of this evaluation was to determine the impact of this free online resource.

Technology

GoToWebinar (Citrix) was used to record conferences. These video files were hosted on a Microsoft Media Server and made public on the website http://pathologyconference.upmc.edu. Google analytics was utilized to track website statistics.

Design

All weekly conferences, seminars and grand rounds at UPMC were broadcast live to internal users, with the speakers' permission recorded, and archived. Recorded videos were converted to MP4 and WebM (HTML5) files. Links to select archived videos were made available online via the department of pathology website. Conferences were uploaded daily, indexed chronologically, and rendered searchable using a Google Custom Search Engine.

Results

From 2002 to 2016, over 2,500 lectures were broadcast and recorded. For some lectures live discussion sessions and audience polling was included. There was on an average 40 internal registrants per lecture. Of these, 844 videos have been shared online for public viewing. Analytics from January 1, 2005 indicate 69,656 page views and 34,650 online sessions. Average time to load our webpage was 1.51 seconds. There were an average of 9 active daily users and 200 thirty-day active users. Returning users comprised 56% of website traffic. Figure 1 depicts the global location of website visitors (color gradient portrays the number of active sessions). Desktop browsing was the preferred method for viewing presentations.

Conclusions

Web hosting of didactic pathology presentations proved to be a convenient and interactive mechanism to provide remote continuing educational opportunities to all of our department members. Although the availability of these online lectures was not advertised, offering this expert educational content as a free resource to the worldwide pathology community has driven users to visit our departmental website.

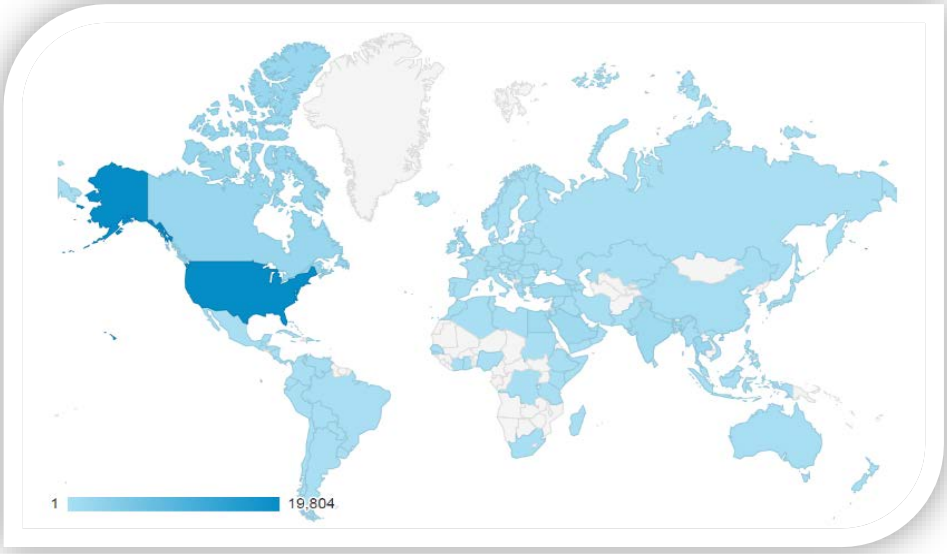

Figure 1. World map showing public usage of UPMC online pathology didactic lectures

Optimizing A Pediatric Pathology Digital Slide Teaching Set with Pathxl^{tm} Tutor Software as an Educational Tool for Pathology Training

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Content
Whole slide digital imaging (WSI) has an important impact on pathology education leading to significant changes in resident/fellow training. The scope of pediatric pathology is diverse, but the implementation of WSI can help narrow the learning gap by ensuring that all trainees see pertinent entities during their short training period. We improved the pediatric pathology WSI teaching set for trainee's education by developing integrated modules with an innovative web-based platform.

Technology
WSI files were captured at 20X resolution using an Aperio ScanScope CS scanner (Leica Biosystems Imaging, Inc., Vista, CA, USA) and uploaded into PathXL^{TM} Tutor software (PathXL Ltd., Belfast, United Kingdom).

Design
A set of 217 scanned glass slides hosted on a department server was accessible using Aperio Webscope (Leica Biosystems Imaging, Inc., Vista, CA, USA) with a separate Microsoft Excel file containing associated clinical information/diagnoses for trainee review. These WSI files were copied to the PathXL image server and integrated with relevant case material using PathXL^{TM} Tutor software.

Results
Thus far, 100 cases are organized into 18 subtopics. Each case module contains a clinical vignette and 1 to 13 WSI including H&E, immunohistochemical, and special stains. The final diagnosis and educational content are revealed after clicking the diagnosis button. Selected cases also contain gross pictures and embedded broadcasted lectures, videos, or PDF articles for further study (Figure 1). Users are allowed to make annotations on WSI files and save them into their personal profile for future review.

Conclusions
An integrated web-based resource that facilitates the use of WSI to supplement traditional teaching methods provides educational benefits for pathology trainees. PathXL^{TM} Tutor serves as an ideal platform to offer trainees consistent exposure to diverse entities in pediatric pathology. The benefit of PathXL^{TM} Tutor includes easy access to web-based digital teaching sets integrated with relevant educational material and resources. PathXL^{TM} Tutor is being explored for the creation of test modules and as a competency-assessment tool in residency and fellowship training.

Expanding the Next Generation Sequencing (NGS) workflow to include Confirmatory Testing

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Content
Next Generation Sequencing (NGS) is becoming more systematically used in the clinical diagnostic laboratory, for somatic and germline genetic mutations. One of the benefits of many NGS platforms is the ability to multiplex not only the tests but the number of samples being analyzed. Many LIMS systems have developed workflows that can be followed for sample management during the NGS process, these workflows often stop at the point of data analysis or right after with a result obtained based on the NGS test. However this is often not the last step of the diagnostic process for the sample, as many NGS test results require confirmatory testing after data analysis.

Technology
NGS workflows are inherently multidisciplinary and rely on the inputs of many departments, which in turn, can utilize a large array of equipment, tools and other software in addition to a LIMS. All such entities communicate to provide a unified technological ecosystem that supports the complexities of a NGS workflow. In this case STARLIMS as part of that ecosystem, manages patient and family information with relevant samples received, prepared and processed before, during and after testing. It manages short and long-term storage of biospecimens and provides the outcome to requesting healthcare professionals.

Design
NGS workflows were designed to follow samples that become multiplexed into single runs, and de-multiplexed for result analysis. The workflows were extended to include reflex testing based on results obtained after result analysis. Confirmatory/Reflex tests were grouped by specific methodology, example Sanger Sequencing Parameters, FISH Parameters, and PCR Parameters. Samples that contained results which required Confirmatory/Reflex testing were queued into the appropriate test group and were thus available.

Results
Prior to including confirmatory testing into the LIMS workflow, samples that needed confirmatory testing, required the manual set up and batching of these test and samples. This could result in delays, and errors especially in high throughput laboratories. The inclusion of multiple possibilities of Confirmatory/reflex testing allowed for comprehensive sample tracking through the entire laboratory process and allowed to include the results of both NGS result analysis and confirmatory test results in the sample history.

Conclusion
Consolidating an NGS workflow with confirmatory testing into one workflow in the LIMS optimizes the laboratory process, reduces the need for documentation redundancies and increases the overall efficiency of tracking samples undergoing NGS testing in a clinical diagnostic laboratory.

Multiplatform Virtual Reality Pathology

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Yale University
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Content

New consumer virtual reality (VR) platforms provide intuitive control mechanisms for interacting with and viewing three-dimensional (3D) data. Multiphoton microscopy and optical tissue clearing allow generation of high-resolution 3D image stacks potentially providing new perspectives on histology. We have designed a VR application for viewing realistic renderings of tissue samples constructed from stacks of multiphoton microscopy images. The application was built to be compatible with both *Oculus* and *HTC Vive* so that their performance could be compared in the context of viewing and analysis.

Technology

The application was developed using Unity 5.5, a popular game development engine, along with libraries for integration with both the *HTC Vive* VR headset and the *Oculus Rift* with *Oculus Touch* controllers. We used a high performance computer with a dedicated graphics unit (Nvidia GTX 1080) and 32 GB RAM to test the application.

Design

The application was iteratively developed based on feedback from testers and pathologists. The tissue is rendered as a 3D cube using stacks of semi-transparent images along each axis. A hematoxylin and eosin transfer function may be applied to the data to simulate staining; the contrast and transparency of the images can also be altered in-application. The cube is displayed stereoscopically through a VR headset. Using motion controllers, the user may translate, rotate, and slice the data along the axis.

Results

A variety of kidney and prostate samples were examined using the two virtual reality devices and the application. While the *HTC Vive* allows for room-scale, 360 degree tracking, this was found to be less relevant in the context of examining pathology samples. The *Oculus Touch* controllers provide additional button inputs for control and better tracks the individual fingers of the user. The performance of the application on both systems was comparable.

Conclusions

This technology allows pathologists to view renderings of histology data in VR. It provides unique views of the surface and structure of 3D samples which are unachievable using traditional 2D microscopy. Further study comparing the effectiveness of our virtual reality technique to more traditional methods of viewing tissue samples is warranted.

Immunoprofiling of Lung Cancer Tissue Specimens Using Multiplex Immunofluorescence and a Multispectral Imaging Platform.

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Departments of [1]Translational Molecular Pathology, [2]Thoracic/Head and Neck Medical Oncology, [3]Pathology, [4]Biostatistics and [5]Melanoma Medical Oncology. The University of Texas MD Anderson Cancer Center, Houston, Texas and [6]PerkinElmer, Inc., Waltham, Massachusetts.

Content
The advent of cancer immunotherapy has prompted the need of large immunoprofiling analysis of solid tumors. To assess and quantify the tumor associated immune cells (TAICs) and immune checkpoints in lung cancer, we optimized a multiplex immunofluorescence (mIF) technique that can be applied to formalin-fixed paraffin-embedded (FFPE) tissues.

Technology
The slides were stained using Opal™ Kit (PerkinElmer, Waltham, MA, USA), scanned using a Vectra 3™ multispectral microscope (PerkinElmer) and analyzed using InForm™ 2.2.1 software (PerkinElmer). The final data was consolidated using SpotFire™ software (TIBCO Software Inc., Palo Alto, CA, USA).

Design
Nineteen immune markers were optimized for mIF using standard chromogenic immunohistochemistry in control, and then we constructed four mIF panels: Panel 1: AE1/AE3 (pancytokeratins), PD-L1, PD-1 (or CD4), CD3, CD8 and CD68; Panel 2: AE1/AE3, CD20 (or PD-1), CD3 (or Granzyme B), FOXP3, CD45RO and CD57; Panel 3: AE1/AE3, PD-L1, B7-H3, B7-H4, IDO-1, VISTA and CD3; and Panel 4: AE1/AE3, ICOS, LAG3, TIM3, OX40, CD3 and CD20. FFPE tissue microarrays (TMAs) contains 256 tumors (adenocarcinomas, ADCs=156; squamous cell carcinomas, SCCs=100), 108 whole sections (WS) specimens (ADCs=61; SCCs=48) and 30 core needle biopsies (CNB) specimens (ADCs=15; SCCs=15) were analyzed.

Results
Positive PD-L1 expression (>5%) in malignant cells (MCs) was observed in 23% ADCs and 31% SCCs in TMAs. 39% ADCs and 56% SCCs in WS showed higher expression of PD-L1 in chemotherapy treated than chemo-naïve cases. Furthermore, CD3+CD4+ (P=0.0030) and CD57+CD45RO+ (P<0.0001) were higher in ADCs than SCCs in all specimens. Positive significant correlation was found when we compared WS and CNB. Panel 3 that included PD-L1, B7-H3, B7-H4 and IDO-1 in TMAs showed that 20% of cases were negative in MCs for all markers, 25% expressed only one marker, 30% expressed two markers, and 25% expressed more than three markers. TAICs expressing ICOS, LAG3, TIM3 and OX40 in panel 4 showed the intricate interactions between T cells (CD3+) expression and immune checkpoints expression.

Conclusion
The advantage of this technique is based on its multiplex approach generating more data per sample and allowing the co-localization of markers. The next step will be to utilize an automatic staining device for efficient mIF.

Optimization of an efficient algorithm for immunoprofiling of lung cancer using InForm image analysis software.

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Content
Immunoprofiling analysis using quantitative immunohistochemistry of tumor-infiltrating lymphocytes and immune checkpoints has been helpful for predicting responses to treatment in many types of solid cancer, including lung cancer. Recently, multiplex immunofluorescence (mIF) has emerged as a powerful tool for immunoprofiling analysis, offering simultaneous detection of multiple markers. To perform the efficient analysis of immunoprofiling using mIF, we optimized an algorithm for InForm™ (PerkinElmer, Waltham, MA, USA) image analysis software.

Technology
Twenty-one lung cancer cases and one tonsil case as a positive control were stained using Opal™ 7-color Kit (PerkinElmer), scanned using a Vectra 3™ multispectral microscope (PerkinElmer) and analyzed using InForm™ 2.2.1 software. The final data was consolidated using SpotFire™ software (TIBCO Software Inc., Palo Alto, CA).

Design
Ten immune markers were optimized for mIF using standard chromogenic immunohistochemistry as a control. We designed two mIF panels with these markers: Panel 1: AE1/AE3 (cytokeratins), PD-L1, PD-1, CD3, CD8 and CD68; Panel 2: AE1/AE3, CD20, FOXP3, Granzyme B, CD45RO and CD57.

Results
Using the spectra of the individual fluorophores extracted in singly stained tonsil sections, as well as an autofluorescence spectrum from an unstained tissue, multispectral libraries for two panels were constructed. Based on these libraries, the unmixed signals were extracted from mIF sections and reconstructed into composite images. To automatically divide each image into two tissue compartments: tumor and stroma, a pathologist trained the software by drawing representative training regions based on cytokeratin staining. Following tissue segmentation, cellular segmentation was performed using DAPI as a counterstain to identify the location of all cell nuclei. Cytoplasmic and embrane subcellular compartments were also determined based on the signal thresholds decided. Once individual cells were identified, the quantification and colocalization of immune markers were achieved according to the intensity of each signal in the trainable phenotyping session. The data created with InForm were also exploited to calculate the distance between tumor and inflammatory cells using RStudio (RStudio, Inc., Boston, MA, USA, https://www.rstudio.com/).

Conclusions
This algorithm offered accurate, reproducible and high-throughput data regarding the tumor microenvironment, when it was used carefully under the supervision of pathologists.

Immunohistochemical Expression of Programmed Cell Death Ligand 1 in Non-Small Cell Lung Cancer evaluated by Digital Image analysis: A Comparison Study between Tissue Microarray and Whole Section

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Content
Programmed cell death ligand 1 (PD-L1) is a major immune checkpoint protein and its expression is used for optimal patient selection for anti PD-1/PD-L1 therapies. Tissue microarray (TMA) technology has been widely used for the immunohistochemical (IHC) analysis of biomarkers in cancer research. However, its reliability for the assessment of PD-L1 expression has not been clarified. Our aim was to compare the scoring reproducibility of IHC PD-L1 expression between TMA and their whole section (WS) counterparts in NSCLC.

Technology
The immunostained WS and TMA were digitally scanned using the Aperio® ScanScope AT2 scanner (20×) and were visualized using ImageScope™ software. The IHC PD-L1 expression was evaluated using digital image analysis (DIA) with Aperio Image Toolbox™ and Genie™ (Aperio, Leica Microsystems).

Design
A TMA set (1 to 3 cores of 1 millimeter per patient) and their WS counterparts of 148 patients with NSCLC were analyzed for PD-L1 expression. PD-L1 expression (E1L3N clone) was evaluated as percentage of positive tumor cells (MCs) by IHC and DIA. Tumor was scored PD-L1 positive when >5% of cells showed membranous staining.

Results
PD-L1 TMA scoring showed a positive correlation with WS scoring (r=0.592, $P < 0.001$). However; the TMA method revealed an overestimation of the PD-L1 positivity comparing with WS (124 vs 55 positives cases respectively, P=0.001). Thus, compared with WS, TMA-IHC had a sensitivity of 93%, specificity of 22% and positive predictive value of 41% for PD-L1. Over-scoring causes in TMA were: misinterpretation of positive macrophages as tumor cells by DIA (63%), PD-L1 tumor heterogeneity (TMA hot spots) (28%) and unspecific nuclear or cytoplasmic PD-L1 expression (6%). The discordance between TMA and WS scoring caused by PD-L1 heterogeneity; decreased significantly when 3 TMA cores per case were evaluated vs <3 cores (10% vs 33%).

Conclusion
IHC PD-L1 expression analysis in NSCLC revealed important discordance between TMA and WS due to misinterpretation/misidentification of staining and cancer cells by DIA and the PD-L1 tumor heterogeneity. To avoid the heterogeneity issue is highly recommended to evaluate at least three TMA cores to obtain a better full section representation. DIA represents an important tool for IHC analysis but requires the pathologist supervision.