

Development of Feature Analysis on Consecutive Tissue Sections (FACTS)

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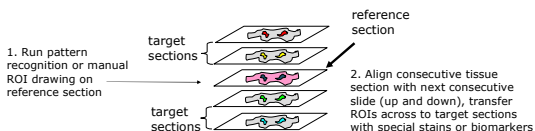
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Introduction

Despite recent advances in histology pattern recognition, automated image analysis is still out of reach for many common pathology applications in the pharmaceutical industry. Frequently there simply is not enough information in the stains utilized in most IHC and H&E studies to provide reliable pattern recognition results without substantial manual curation post analysis. In addition, there remains an unmet need for multiplexing in IHC in brightfield applications beyond one or two proteins.

We have developed a patent pending approach to both pattern recognition and multiplexing called Feature Analysis on Consecutive Tissue Sections (FACTS). The process involves utilizing a target tissue with strategically chosen stains for the region of interest. We utilize careful serial sectioning of thin 4 μm sections with one or more target sections on either side of the reference section, that are stained for the biomarker or lesion to be measured. The regions of interest are computed once on the reference section and then transferred to the target sections by one of several methods from the field of image registration.

In this presentation we look at a number of computational image registration and region of interest transfer approaches from radiology and other areas and present some validation results for several histology applications. Each registration approach has its advantages in certain biological applications. The FACTS method requires only minimal changes to current histology practice, and the labor costs are less than what is required for running multiple IHC markers in clinical or preclinical laboratories. Validation for both GLP or CLIA can be done without substantial modifications to common histopathology laboratory practices.



Process for Feature Analysis on Consecutive Tissue Sections (FACTS)

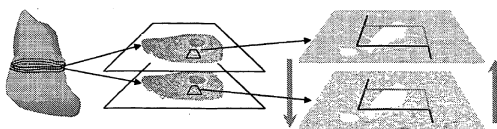
Multimodal, Semi-Automatic Image Registration

Image registration for biomedical applications has a long history in biomedical applications. Because FACTS generally uses different stains between the target and reference tissue, it is related to other multi-modal biological imaging problems like brain CT/MRI images, whole body tumor localization PET/CT images, contrast versus non-contrast-enhanced CT images, or ultrasound/CT image registration (e.g prostate radiotherapy). In FACTS, we utilize a reference section (or sections) with a different stain than that used in the target sections. This could be an H&E, PAS, or other stains commonly used by pathologists. It could also be a special immunostain for identifying specific cell or tissue types to aid in tissue pattern recognition. This presents specific challenges to the well established field of image registration.

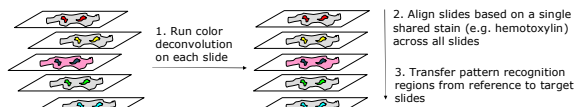
Because each section is typically 4 μm apart, there are biological differences between sections. We thus employ a morphing approach to the ROIs themselves, combined with a semi-automatic approach with user interaction (e.g. pathologist supervision and ability to rapidly modify a given transferred region)

Multiple registration approaches required

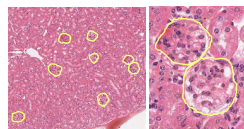
The problems encountered in our FACTS analysis service are diverse, and range across discovery, preclinical toxicology, and oncology clinical trials. We thus utilize a number of approaches to image registration as outlined below.



In stereology above (Visiopharm, Denmark) the tissue areas in two images are detected, and at low magnification a rigid warp of one images is fitted approximately on the other, followed by a non-rigid warp based on approximate fit. The images are then viewed at high-magnification, and analysis algorithms on one registered image can be applied on the other. In this approach, we prefer to utilize the warped image as the reference image, and the non-warped image as the target image, to avoid computationally changing IHC or other image data during the warping process. This method works well for images with similar staining approaches and where significant warping of tissue between consecutive sections has occurred.



In many FACTS applications, we utilize a reference slide with a different stain than the target slides. We may use H&E for the reference slide, or a tumor/stromal differential stain, or an anatomical stain (e.g. glomeruli, islets, bronchiole, brain anatomy, etc). The target slides are frequently IHC slides. In the approach developed by Flagship, we utilize color deconvolution on all slides, with a commonly shared stain (e.g. hemotoxylin) used to align and register the slides.



Once images have been aligned and regions transferred and morphed from pattern recognition on the reference slide, our pathologists can then further translate, rotate, shrink, expand, or use a magic pen tool to snap to obvious feature lines to overwrite the automated analysis if necessary

Our in-house approaches are semi-automatic. The images are registered using an approach as mentioned above. Once regions on the reference slide are found with pattern recognition, they are vectorized, and then mapped and morphed onto the target slides and presented to the Flagship pathologists or technicians for review.

Biological and algorithm error estimation

Errors in the FACTS process arise from three sources: 1) automated feature recognition (AFR) on the reference slide, 2) registration alignment errors to the target slide, and 3) variability in the features between the reference and target slides. The use of special stains for individual FACTS applications on the reference slide reduces the first error. Registration alignment is reduced by the use of color deconvolution if needed and a common stain shared across all slides. The third error is challenging. Plastic histology embedding allows for <3 μm sections but this has proved expensive, and violates our goal of an approach that can be run in a competent histology laboratory. There is no scalable approach to separating the second and third error type, so in our validation processes we simply measure the error between the transferred region of interest and the target slide as part of our in-house quality control processes (shown below). Some of these error rates are shown below. As expected, they increase with decreasing feature size or higher tissue variability between sections.



To estimate error per feature (as in this glomeruli example), we first must map the transferred region from AFR as well as find the "correct" region. In the validation study, the "correct" region is drawn manually by a pathologist.



The differences between the two regions (XORed area) is then divided by the mapped region to give false positive, false negative, and total error per feature

In many applications, false negative errors have little impact. It is only necessary to ensure measurement occurs in only target tissue, not that all target tissue is measured. This includes endpoints like:

- Xenograft neoplasm protein expression & angiogenesis
- ER/PR/HER2 measurement in neoplasm regions
- Biomarker expression in neoplasm
- Beta/alpha cell mass in diabetes
- Glomerular analysis in nephrology & toxicology

| Feature | Average size | False positive | False negative | Total error |
|--------------------------------------|--------------|----------------|----------------|-------------|
| Liver bile ducts | 56 μm | 0.6% | 4.1% | 4.7% |
| Kidney glomeruli | 65 μm | 0.9% | 4.9% | 5.8% |
| Fibrous capsule in implants | 62 μm | 1.3% | 1.4% | 2.7% |
| Pancreas islets | 135 μm | 1.0% | 3.8% | 4.8% |
| Xenografts (H&E to CD31 stains) | 490 μm | 0.6% | 0.4% | 1.0% |
| NCSLC clinical trials samples | 315 μm | 1.5% | 1.7% | 3.2% |
| Spleen periarterolar lymphoid tissue | 520 μm | 0.3% | 0.7% | 1.0% |

Multiplexing protein expression

The limit on protein multiplexing is largely determined by the third error source, the variability in the features between the reference and target slides as the distance grows further away. We are working on a stair-stepping image registration approach, where the adjacent target slide is aligned to the reference slide, and then the next adjacent target slide is aligned to the annotations from this target slide. This would allow as many as 4-6 biomarkers to be measured. Another approach is to use IHC dual-staining on the target slides, but we have not found dual-staining to be reliable and robust enough for regulated pathological examination, particularly where some degree of semi-quantitative assessment is required.

Advantages in regulated pathology

Flagship Biosciences is currently utilizing FACTS in discovery services with drug and device clients, with plans for oncology clinical trials and preclinical toxicology assessment. There are several advantages to FACTS over other multiplexing approaches:

- Utilizes brightfield – only brightfield IHC is FDA-cleared for digital imaging applications in protein expression
- No special histology processes are required, most high-quality histology labs can process 4 micron consecutive sections
- Complete glass and tissue record of all multiplexed tissues – no re-staining of any slides. This is extremely important for regulatory compliance aspects (e.g. 21 CFR 11 and 58)
- Inexpensive histology processes. The complexity is in the image registration and software.