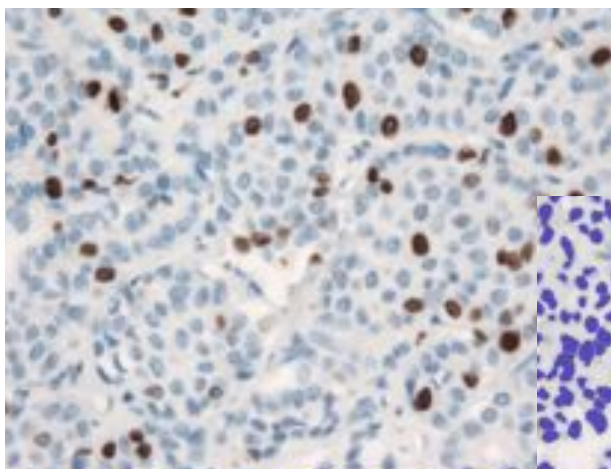


ImmunoRatio-F: image analysis of Ki-67 using cytokeratin immunofluorescence correction

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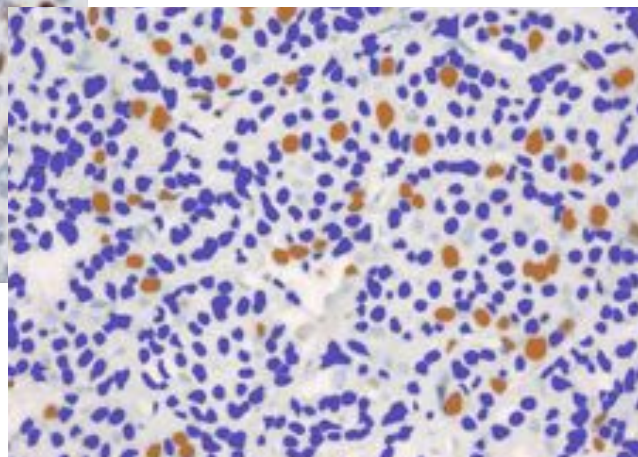
**Accurate reporting of ER,PR, and Ki-67
is clinically important (% pos. cells)**



original Ki-67 staining

ImmunoRatio software:

Ki-67 labeling index by
(=brown / blue+brown area)



pseudo-colored analysis result

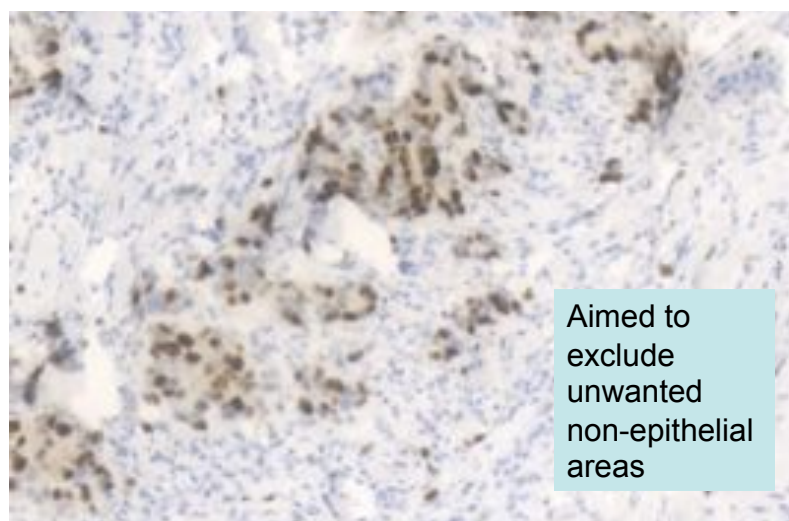
In 2010 we launched ImmunoRatio
as a freely available web application



It has become popular !!

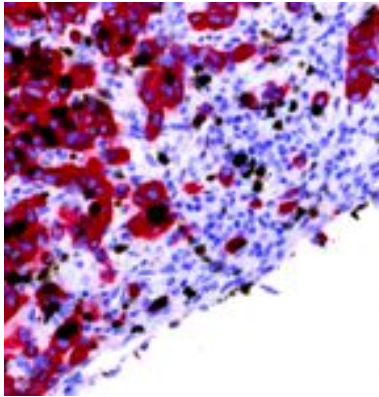
Tuominen V et al. Breast Cancer Res 2010,12(4), R56

ImmunoRatio-F is an improved version of ImmunoRatio

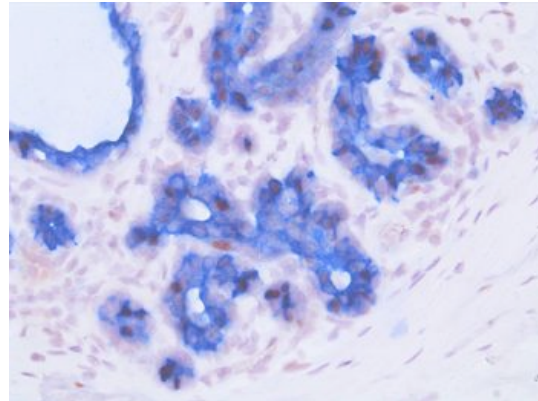


Non-epithelial cells lower (dilute) the Ki-67 LI

Solution #1 for epithelium specific image analysis : double staining



cytokeratin+Ki67+hematox



cytokeratin+Ki67+hematox

Ki-67 negative nuclei show up poorly !!! Difficult to quantitate

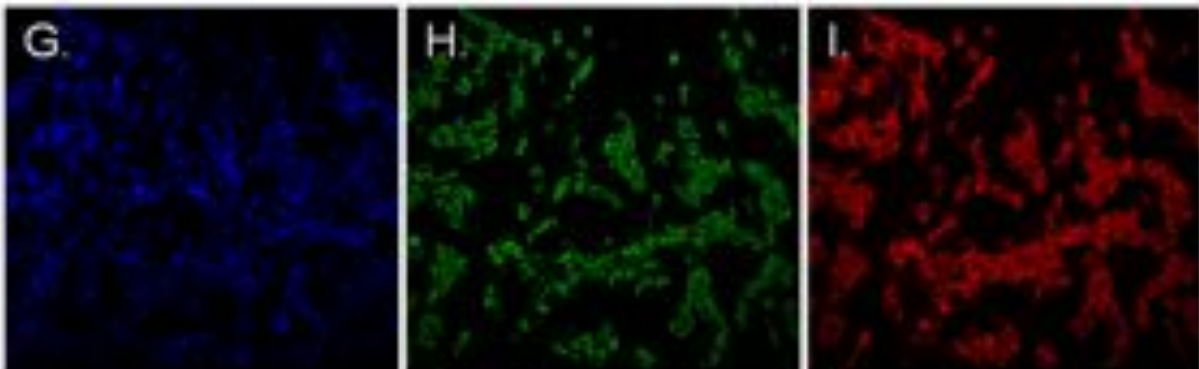
Two chromogenic signals are messy.

Solution #2 for epithelium specific image analysis : double immunofluorescence

DAPI / nuclei

Cy3 / cytokeratin
tumor mask

HER-2



AQUA / HistoRx is a well-known product for this application

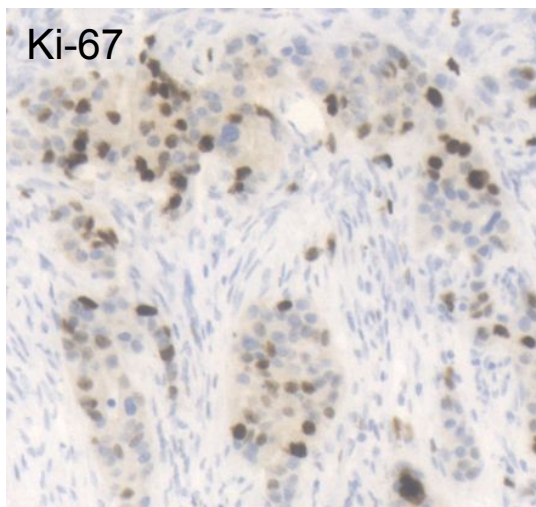
But: most pathologists are reluctant to spend time with fluorescence microscopy.

Let's take best of both worlds: combine DAB-IHC and fluorescence

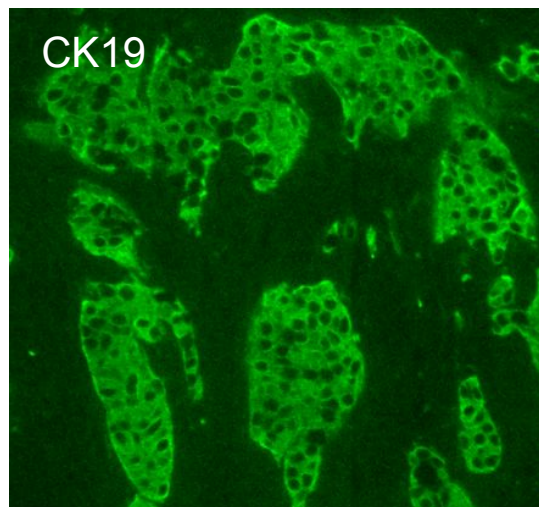
Staining protocol needs only three minor modifications:

- 1) add mouse anti-CK19 to rabbit primary antibody (= make antibody cocktail)
- 2) add anti-mouse DyLight488 to anti-rabbit HRP polymer (=make detection reagent cocktail)
- 3) Mount slides with antifade (Pro-Long Gold)

Chromo-fluorescent double IHC stain



Brightfield Ki-67 layer
- looks similar to 1-color IHC
- every pathologist is familiar with



Hidden cytokeratin layer
- not inspected by the pathologist
- images captured automatically

IHC double stains are based on mouse/rabbit antibody pairs

- Anti-rabbit HRP+DAB detects rabbit primary ab (Ki-67 clone SP6)
- Anti-mouse DyLight488 detects CK19 (clone 603, Jilab Inc)
- Pairs can be formed also vice versa

Mouse and rabbit antibodies detected specifically with secondary antibodies.

Useful mouse & rabbit antibody pairs

DAB-stained ab	fluorescence mask ab	indication
Ki-67 (clone SP6)	CK19	Breast & other carcinomas
ER and PgR (clones SP1 and SP2)	CK19	Breast
Ki-67	AMACR	Prostate
Ki-67	CD3 / CD20	Lymphomas
HER-2 (clones SP3, EP1045 etc.)	CK19	Breast, stomach
Ki-67	Melan A	Melanoma

Many motorised microscopes and scanners can capture brightfield and fluorescence images sequentially



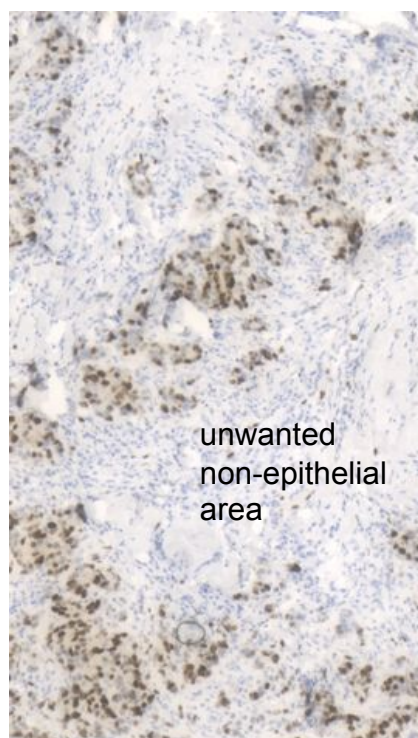
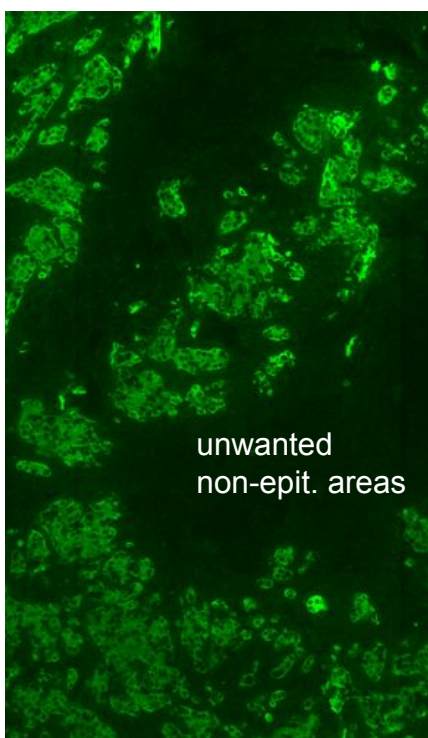
3D Histech Panoramic



Leica DM4000 B

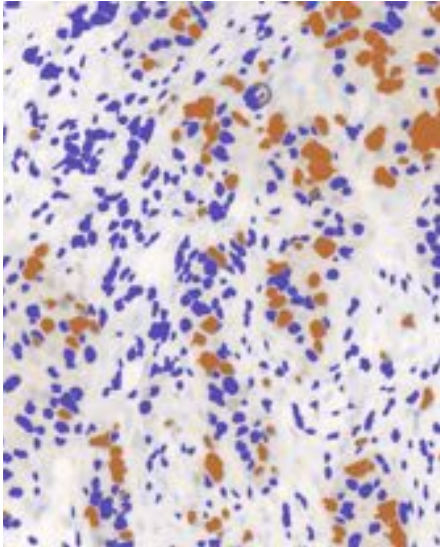
No need to inspect fluorescence by eye

Cytokeratin mask correction useful in tumors with dense cellular stroma and/or lymphoid infiltrate

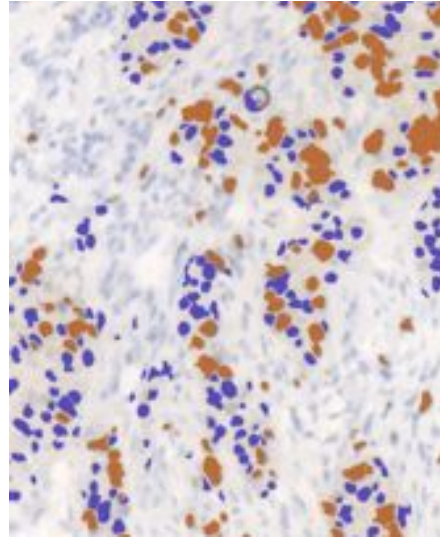


ImmunoRatio-F output with and without cytokeratin mask correction

pseudocoloured analysis results (Ki-67 and hematox)

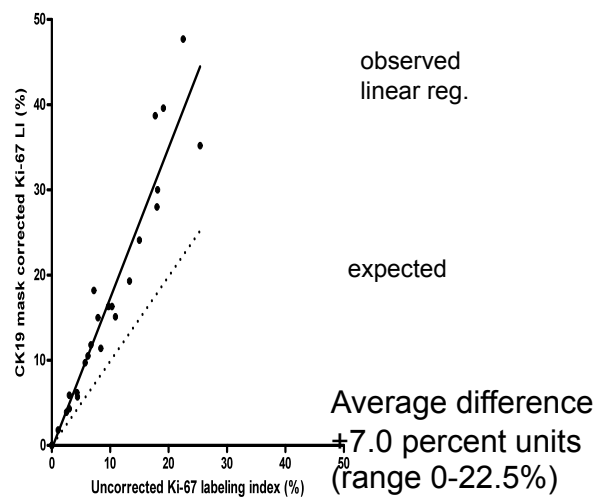


without correction
Ki-67 LI = 23%



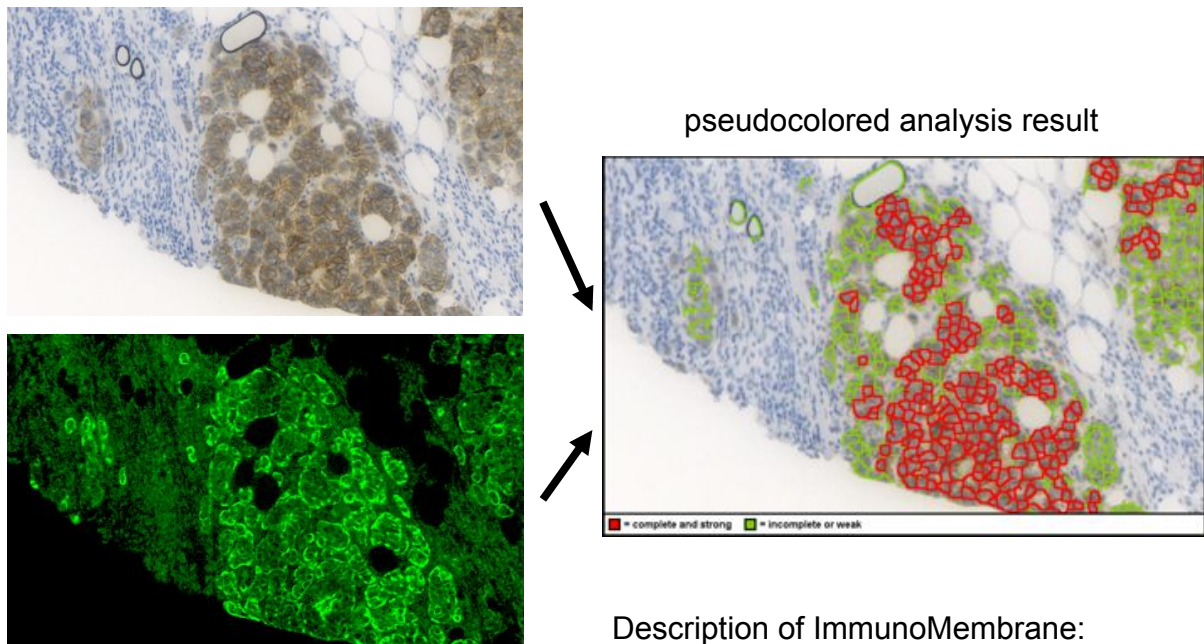
with CK19 correction
Ki-67 LI = 47%

Ki-67 labeling index with and without cytokeratin mask correction



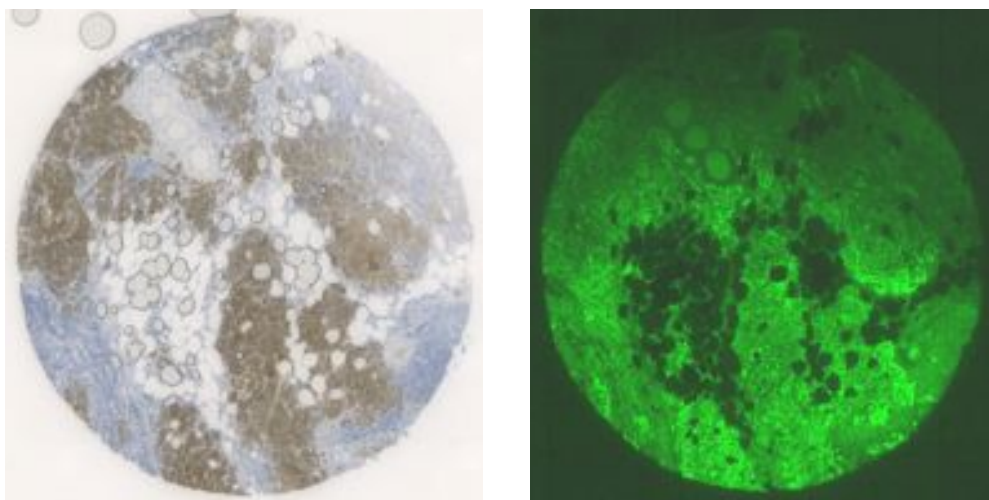
Ki-67 labeling indexes from 25 consecutive breast cancers

Cytokeratin 19 masking applied to HER-2 IHC image analysis (ImmunoMembrane software)



Description of ImmunoMembrane:
Tuominen V et al. Histopathology
2012 Apr;60(5):758-67

CK19 mask correction allows automated analysis of tissue microarrays



Analyses restricted to epithelial cells
-- compensates TMA sampling bias

Conclusions :

- Chromo-fluorescent double IHC is a new way to improve accuracy of image analysis
 - Ki-67, ER, PR and HER-2 tested so far
- Can be applied to routine diagnostics with minor changes in staining and image acquisition

See <http://jvsmicroscope.uta.fi>