S60 Hamamatsu Scanner: Upgrading to Six Simultaneous Fluorochromes for Tissue Imaging

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Multiplex immunofluorescent staining routinely processed tissue has become the state-of-the-art technology to investigate biology and pathology at the single cell level. Routinely processed formalin-fixed, paraffin embedded (FFPE) tissue is the most available and robust material for such applications.

Tissue sections may produce autofluorescence signals [1], depending on the tissue type and the processing (fixation and embedding); in general, tissue autofluorescence is maximal on the left side of the spectrum and almost nil in the red part (>600 nanometers; nm) [1]. Such signals are a nuisance and interfere with the acquisition of a specific signal by an exogenous fluorescent probe (antibody, DNA probe).

Tissue autofluorescence can be collected by combining mismatched filter combinations as exemplified in Figure 1 and 2 and digitally subtracted from the image carrying the specific marker as published [2].

Two separate requirements prompted the search to broaden the fluorescent imaging capability of the NanoZoomer S60 scanner:
A- the need for tissue imaging in an emission range with minimal tissue autofluorescence and B- the need for more simultaneous, separate fluorochromes in a multiplex staining application [2].

The Hamamatsu NanoZoomer S60 slide scanner is one of the few commercial slide scanners which allows imaging in immunofluorescence of multiple slides (>5), with at least three fluorescence wavelengths (DAPI, FITC, TRITC) [NOTE: the names of reference fluorochromes, e.g. FITC, TRITC, Cy5 etc., will be used throughout this manuscript in lieu of the actual fluorochromes or conjugates used by individual users].

The Hamamatsu NanoZoomer S60 scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan) is equipped with a Nikon 20×/0.75 PlanSApo objective, a Fluorescence Imaging Module equipped with a L11600 mercury lamp (Hamamatsu Photonics K.K.), a linear ORCA-Flash 4.0 digital CMOS camera (Hamamatsu Photonics K.K.) and two six-position filter wheels, one for excitation, the other for emission filters, and a three-cube turret. The current filter setup at UNIMIB [Figure 1] is composed of a set of excitation filters, housed in one wheel: 387/11 (DAPI), 438/24 (BV480), 480/17 (FITC),

	Fluorochrome	excitation	emission	dichroic
DAPI	DAPI	FF01-387/11	FF02-435/40	FF403/497/574-Di01
Brilliant Violet	BV-480	FF02-438/24	FF01-483/32	FF458-Di02
FITC	Alexa 488	FF01-480/17	FF02-520/28	FF403/497/574-Di01
TRITC	Rhodamine-RedX	FF01-556/20	FF02-617/73	FF403/497/574-Di01
Cy5	Alexa 647	FF01-650/13	FF01-673/11	Di02-R635
PerCp	PerCp	FF01-480/17	FF01-698/70*	Di02-R635
AF		FF02-438/24	FF02-617/73	FF403/497/574-Di01
AF2		FF02-438/24	FF01-483/32	FF458-Di02
AF3		FF01-480/17	FF02-617/73	FF403/497/574-Di01

AF: autofluorescence, AF2 and AF3 filters were used when no BV-480 or only FITC/TRITC/Cy5 were used, respectively.
Housed 35 mm. excitation and emission filters were allocated in two 6-filter wheels; dichroic filters were housed in three filter cubes (Olympus).
* do not use this filter for Cy5 acquisition: the excitation light will bleed into the acquisition filter set. An alternative for dual Cy5/PerCp use is the FF01-682/24.

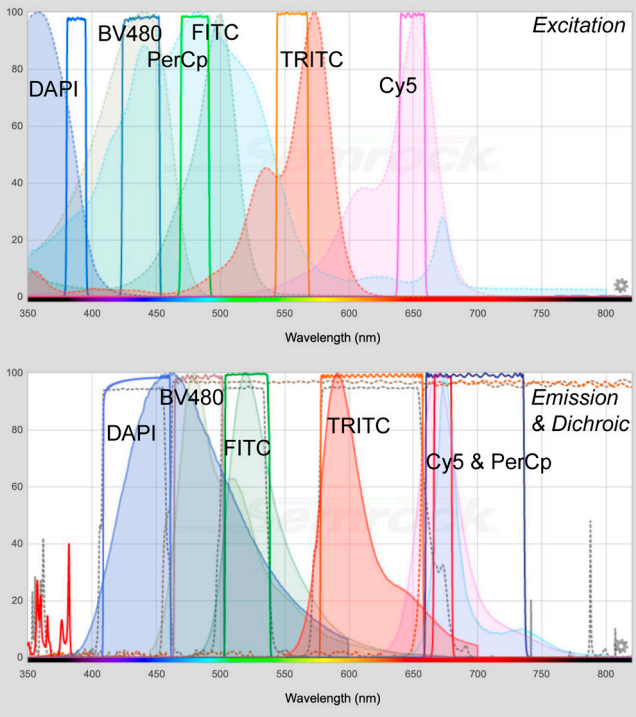

Figure 1: Hamamatsu NanoZoomer S60 filter setup for six color + AF acquisition.
Top panel: the fluorochrome conventional name, actual fluorochrome and filter specifics for excitation, emission and dichroic filters are listed and color-coded. Three separate autofluorescence (AF) acquisition setup via filter mismatch are also illustrated.
Bottom panel: excitation and emission profiles for the fluorochromes and the profile of the filters are plotted on a fluorescence scale. The graphs are from the SearchLight Semrock website.

556/20 (TRITC), and 650/13 (Cy5); one set of emission filters: 435/40 (DAPI), 483/82 (BV480), 520/28 (FITC), 617/73 (TRITC), and 694/44 (Cy5). Dichroic filters are housed in three OM cubes: a triband FF403/497/574-Di01 (DAPI/FITC/TRITC) and two single pass FF458-Di02 (BV480) and FF655-Di01 (Cy5). All filters are from Semrock, Lake Forest, Ill, USA.

Fluorescent dyes emitting in the 700-800 nm range are not sufficiently excited by the L11600 mercury lamp, thus we did not further investigate their use, despite the broad availability of immunoconjugates.

Emission from the Alexa 647 dye may theoretically be separated from the one for Alexa 680. To test this, we acquired two separate filters, FF01-673/11 for Alexa 647 and FF01-775/140 for the Alexa 680, both housed in the emission filter wheel. Unfortunately, emissions from the two dyes overlap and could not be separated.

Next we tested a series of new dyes, Brilliant Violet [3], characterized by the excitation in the DAPI range (about 400 nm) and emission in the orange/red range. We currently use one of these dyes, BV480, whose filters occupy two positions, one in each wheel and one for the dichroic in a dedicated filter cube in the turret. To test these dyes we first changed the default dichroic filter (FF655-Di01) for Cy5 with another filter (Di02-R635) with greater control of bleedthrough in the blue excitation range [Figure 1]. This filter was housed in the appropriate OM cube. Avidin-BV711 was excited with the FF01-387/11 and collected with the Di02-R635 dichroic and by the FF01-775/140 emission filter, however we could not collect enough specific fluorescence.

We then searched for duochromes, tandem dyes in which one dye is excited at a lower nm range and by fluorescence energy transfer (FRET) excites the other dye, which emits at a considerable right shift [4]. The availability of two filter wheels is of paramount importance to allow a flexible filter combination.

APC/Cy7 is excited by the Cy5 filter set (FF01-650/13 in our case) and emits in the 800 nm range. We acquired an appropriate emission filter (FF01-796/64), which would keep out the APC own emissions, but no specific fluorescence could be collected, probably because of the low excitation energy of the Cy5 excitation filter. In addition, APC was imaged together with Alexa647 with filter combos for the latter. Then we turned to older dyes such as Pe/Cy5.5 and PerCp.

Pe/Cy5.5 is excited by the TRITC filters (FF01-556/20) and the emission is collected by the FF01-650/13 or the FF01-697/58 [Figure 1]; however, when imaging at the same time with the TRITC filter combination Rhodamine-RedX and Pe/Cy5.5, Pe emits together with Rhodamine, making this duochrome not convenient to use.

PerCp instead was convenient for two reasons; it can be collected separately from the other dyes present on the slide. Furthermore, tissue autofluorescence is minimal with the filter combination for it [Figure 2].

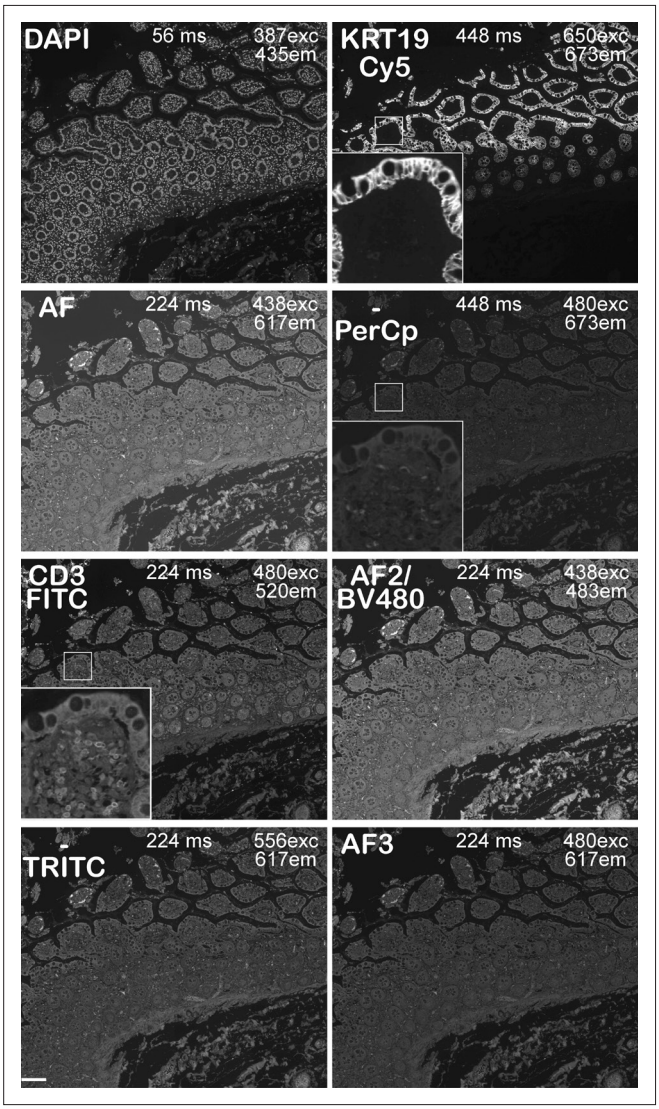

Figure 2: Specific staining and autofluorescence with the filter combinations used. The fluorochrome, the marker (if present), the exposure time in milliseconds (ms) and the excitation and emission filter wavelengths are reported for each of the eight possible combinations. Names of each filter set is as per Figure 1. Note the low tissue autofluorescence for PerCp, exposed at twice the time compared to others. Also notice the absence of bleedthrough for CD3 and KRT18 in the PerCp image (insets). The reverse is also true (not shown). Scale bar = 100 µm.

A good variety of PerCp immunoconjugates are available, which allows the simultaneous assessment of six different colors, plus autofluorescence, providing that the correct combination of mouse Ig isotypes and animal species is arranged, when using indirect IF techniques as we do. As a proof of principle, we immunostained routinely processed human tissue (colon) with five antibodies (Table 1), counterstained with secondary antibodies conjugated with five different fluorochromes (Figure 3) [5] plus DAPI and the autofluorescent channel, for a total of seven images per whole section.

It is thus feasible to selectively illuminate six different dyes with the Hamamatsu NanoZoomer S60, and in addition, subtract tissue autofluorescence, providing greater throughput and flexibility to the instrument.

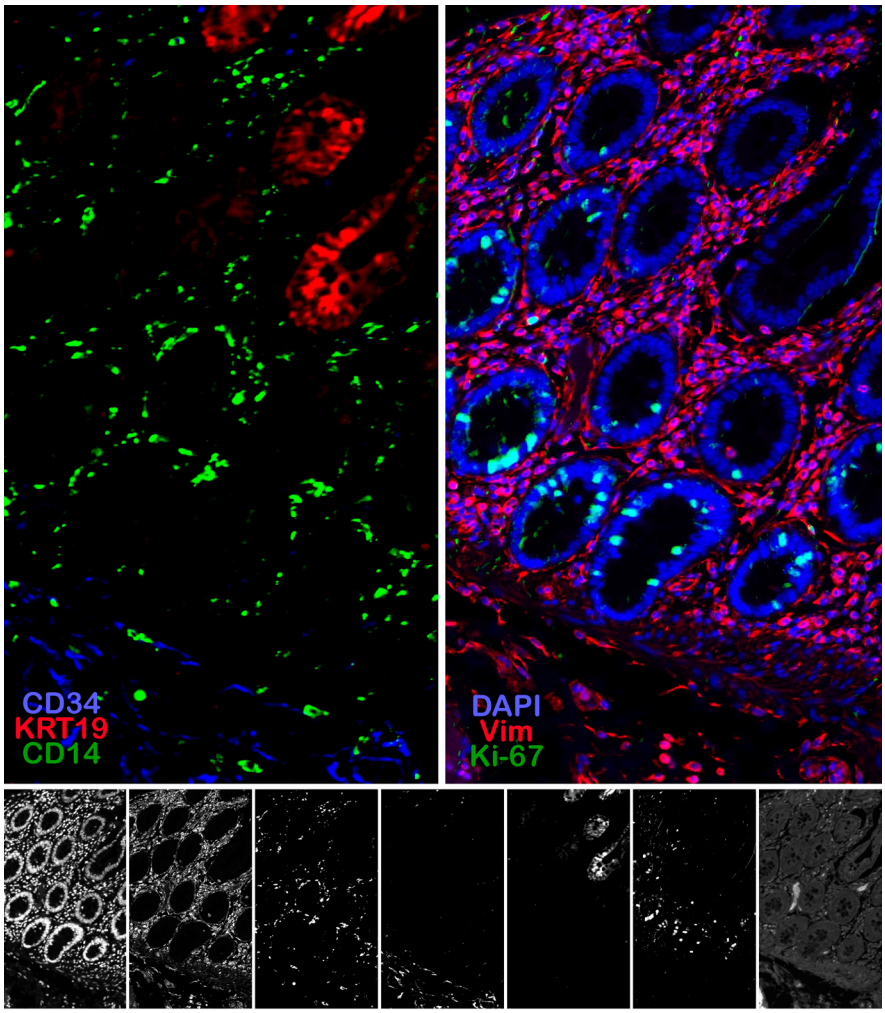

Figure 3: Six color staining of human FFPE tissue. A single whole section from a routinely processed (FFPE) human colon was stained in indirect immunofluorescence for five antigens plus DAPI, images acquired, autofluorescence subtracted and the markers stained in pseudocolors three at a time (top panels). The seven images obtained are shown in greyscale at the bottom, together with the filter combinations used.

References

1. G. A. Wagnières, W. M. Star, B. C. Wilson, In vivo fluorescence spectroscopy and imaging for oncological applications. Photochemistry and photobiology 68, 603-632 (1998).
2. M. M. Bolognesi et al., Multiplex Staining by Sequential Immunostaining and Antibody Removal on Routine Tissue Sections. Journal of Histochemistry & Cytochemistry 65, 431-444 (2017).
3. P. K. Chattopadhyay et al., Brilliant violet fluorophores: a new class of ultrabright fluorescent compounds for immuno-fluorescence experiments. Cytometry Part A : the journal of the International Society for Analytical Cytology 81, 456-466 (2012).
4. A. N. Glazer, L. Stryer, Fluorescent tandem phycobiliprotein conjugates. Emission wavelength shifting by energy transfer. Biophysical journal 43, 383-386 (1983).
5. F. Mascadri, M. M. Bolognesi, D. Pilla, G. Cattoretti, Rejuvenated Vintage Tissue Sections Highlight Individual Antigen Fate During Processing and Long-term Storage. Journal of Histochemistry & Cytochemistry. September 2021. doi:10.1369/00221554211047287.