Comparison of fluorescent imaging systems for increasing multiplex capabilities

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1. Introduction:

Multiplexing fluorescence techniques on tissue sections allow detection of multiple markers whilst simultaneously preserving spatial context. Detection and quantification can be achieved with the use of image analysis software following image acquisition. However, autofluorescence (AF) and spectral overlapping of fluorophores can compromise the accuracy of target detection and analysis performed.

An imaging system capable of generating high resolution images with effective fluorophore unmixing and elimination of background can provide reliable outputs for accurate qualitative and quantitative analysis.

This comparison of different imaging systems is with the aim to:

1. **Increase multiplexing capability** for image acquisition with minimal spectral overlap
2. **Eliminate autofluorescence** interfering with real signal
3. Accurately **identify and analyse different markers simultaneously** in the same tissue section.

2. Methods:

A fluorescent RNAscope ISH multiplexing technique from Advanced Cell Diagnostics (ACD) was used for detection of 4 mRNA probes in formalin-fixed, paraffin-embedded tissue microarrays. Levels of RNA expression were assessed across a range of tissues from mouse.

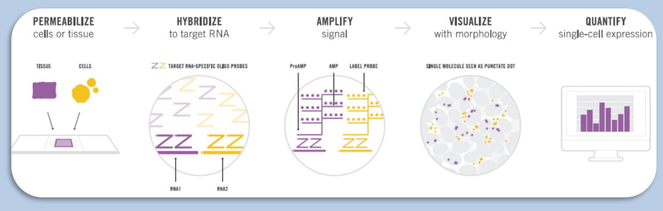


Figure 1 - RNAscope workflow (ACD).

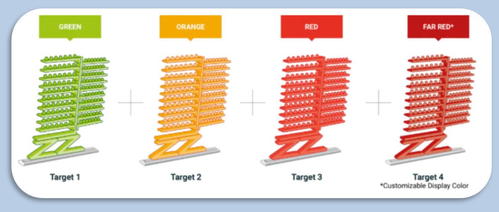


Figure 2 - RNAscope Multiplex Fluorescent Assay: channel specific probe *design (ACD).*

The following imaging systems were selected to assess the markers detection and quantification ability:

	Imaging Systems		
Features	**Axioscan (Zeiss)**	**Operetta (Perkin Elmer)**	**Vectra Polaris (Akoya)**
Multiplexing Capability	Up to 5 colours	Up to 6 colours	Up to 9 colours
Scanning Resolution	Whole slide from 5X to 40X (0.11 µm/pixel)	5X Whole slide to 63X tiles (0.66 µm/pixel)	Whole slide and Multispectral to 40X (0.25 µm/pixel)
Filter sets	Combination of Long and narrow band pass filters	Narrow band pass	Narrow band pass
Exclude AF	Yes, using an analysis classifier	N/A	Yes, using spectral unmixing
Compatibility with image analysis software	Halo	Harmony	InForm, Halo

Table 1 – Imaging systems features.

Staining was analysed using HALO image analysis, with the FISH v2.18 module, from Indica Labs. Average probe copies per µm^2 detected for each probe were exported from Halo.

3. RNAscope Results:

Operetta images could not be compared with those obtained on Axioscan and Polaris due to the practical limitations involved in fusing individual Operetta tiles into a whole slide image. Operetta images showed higher levels of AF compared with the Polaris and Axioscan images. Images obtained with the Polaris have lower lever of AF than the images from Axioscan allowing for better target detection visually.

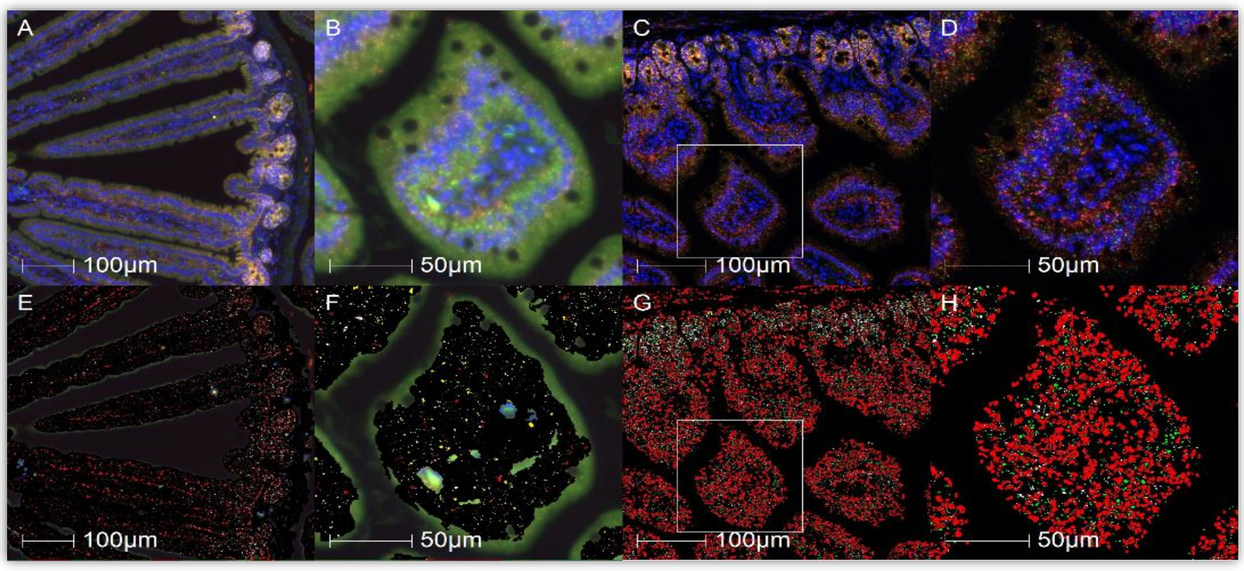
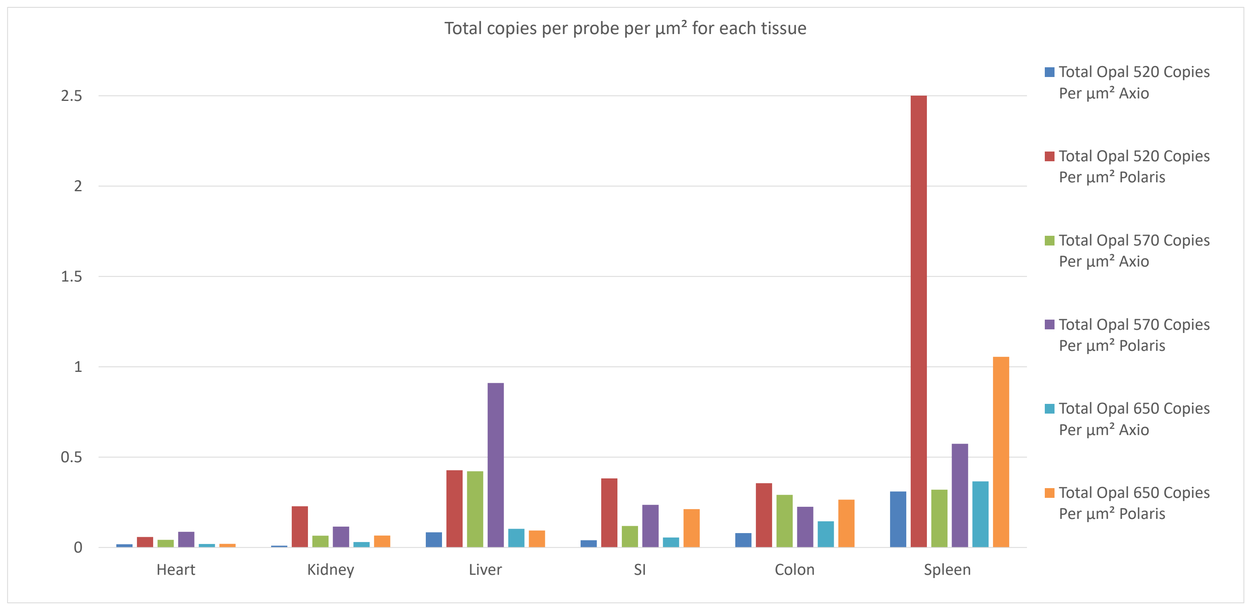


Figure 3 - Comparison between Axioscan and Polaris Colon TMA Core positive control. Images A, B, E, F show mark-up from Axioscan and images C, D, G, H show mark-up from Polaris. A-D. Raw images. E-H Analysis mark-up (A, C, E, G, I – 10x. B, D, F, H, J – 40x).

4. Number of RNA copies detected:

Vectra Polaris demonstrated to have the best performance from the three imaging systems by detecting and quantifying up to four times more probe copies per µm^2 when compared with Axioscan images. A comparable analysis of the probe copies was not possible with the Operetta images as only individual tiles could be analysed.



Graphic 1 - Total number of probe copies detected per µm^2 per Axioscan and Polaris Set for each Tissue Cores. Results are displayed per tissue type and show the total copy number of each probe detected per µm^2 using Halo Software in all the positive controls for each tissue type.

5. Imaging System Results:

Images acquired on Operetta could not be compared with those obtained on Axioscan and Polaris nor could they be analysed in Halo as whole images. Images acquired on Polaris demonstrate higher signal-to-noise ratios than images acquired on Axioscan, with lower levels of AF.

		Axioscan	Operetta	Vectra Polaris
Setup	Protocol setup time	~ 3h	~ 1h	~ 1h (including library creation)
	Preview time	~1h	~1min (5x)	~12 min (10x)
Output	Image acquisition per tissue section	~25h	~3h	~10h (40x)
	Z-stacks	5	10	None
	Image size	8GB	188GB	23GB
	N. tiles (approximately)	~1460	~ 600	~ 450
	File type	czi.	tif.	im3.
Feature	Image Processing	N/A - included in acquisition time	Not possible to perform	~ 8h (including maximum projection and spectral unmixing)
	Ability to fuse images (as part of scanning)	Yes	No	Post-processing in Halo
IA	Probe copies per cell for each target	Yes, using Halo FISH algorithm	N/A	Yes, using Halo FISH algorithm

Table 2 – Summary of features and capabilities measured for each system.

6. Conclusions:

The automated RNAscope ISH assay provides consistent and reproducible results with high signal-to-noise ratio and little background staining. Along with a Multispectral Imaging System, that can efficiently reduce AF and extract the spectral signature for each fluorophore, more accurate image analysis results can be obtained.

Vectra Polaris has demonstrated to have the best performance from the three imaging systems for the acquisition of high quality spectrally unmixed images that can be analysed in our preferred image analysis software.

We have concluded that the purchase of a Vectra Polaris would benefit the Core Facility and Cambridge Institute Researchers by:

- Increasing multiplexing capability
- Allowing high throughput whole slide scanning, applicable to a variety of ISH & IHC methods
- Decreasing our turnaround times
- Improving accuracy of image analysis results

7. References:

- Anderson, C.M., *et al.* Fully Automated RNAscope In Situ Hybridization Assays for Formalin-Fixed Paraffin-Embedded Cells and Tissues. Journal of Cellular Biochemistry 117, 2201–2208 (2016).
- Stack, E. C., *et al.* Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods* 70, 46–58 (2014).
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