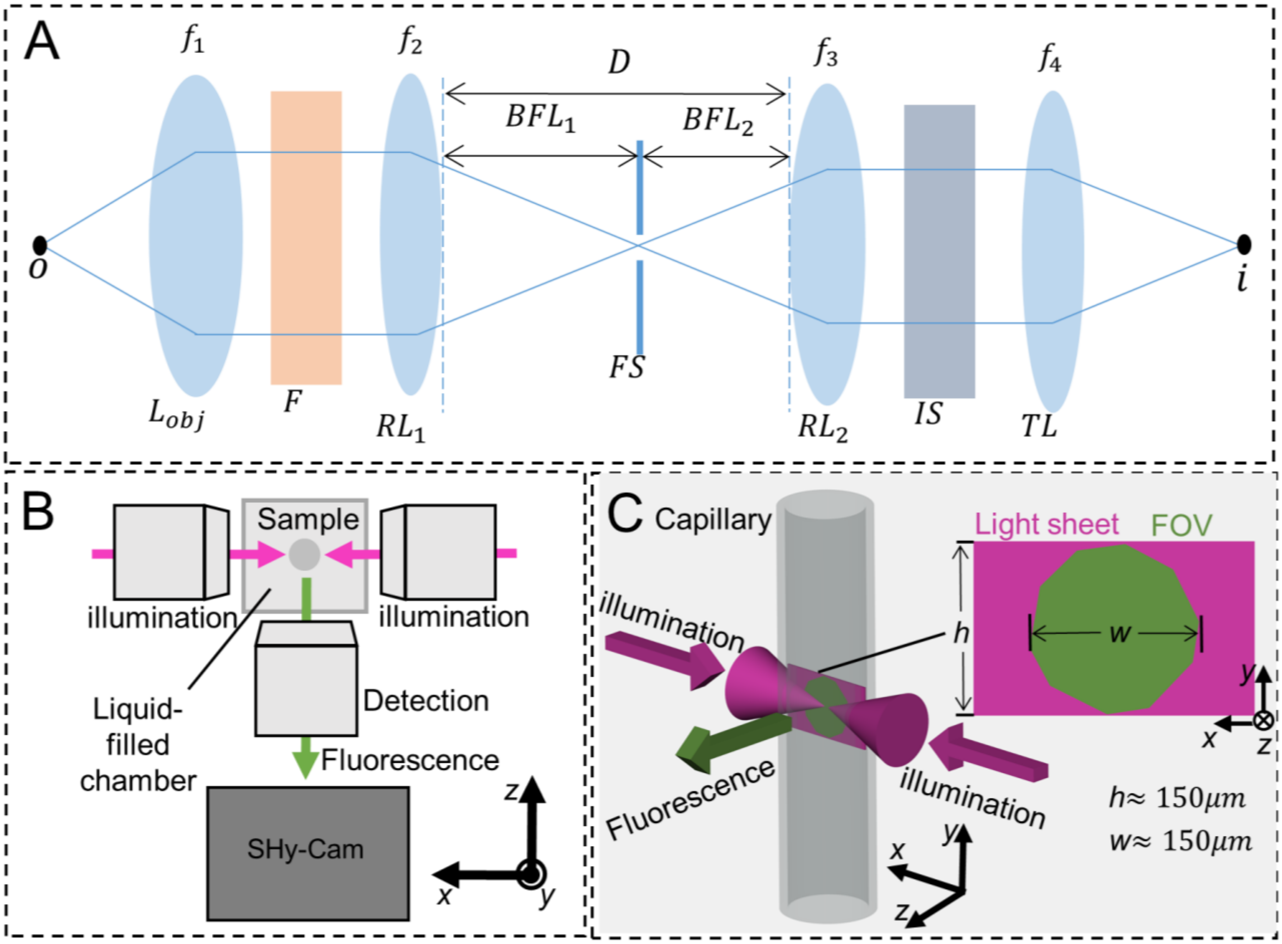

Figure S1 SHy-Cam 3D model and optical diagram of one channel. A The optical train of one channel. *o* represents a point on the object plane (on- or off-axis) and *i* represents its corresponding point on the image plane. L_{obj} is the microscope detection objective lens with effective focal length f_1. F represents a pair of long-pass (FF01-380/SP25, Semrock, Inc) and short-pass (FF01-715/SP25, Semrock, Inc) filters which reject photons outside of 400-700nm working spectral range. RL_1 and RL_2 are a pair of relay lenses with focal lengths of f_2 and f_3. BFL_1 and BFL_2 are the back focal lengths of RL_1 and RL_2 which decide the spacing D between the last surface of RL_1 and the first surface of RL_2. FS is a field stop placed at the intermediate image plane (at a distance BFL_1 after the last surface of RL_1) of the relay lens pair. IS represents the image splitter portion of SHy-Cam (related to Figure 1 and STAR Methods). TL represents a tube lens which converges and focuses the light with an effective focal length of f_4. The SHy-Cam presented in this work uses: $f_1 = 8mm$ (CFI75 Apochromat 25XC W, Nikon), $f_2 = 150mm$ (AC508-150-A, Thorlabs, Inc.), $f_3 = 150mm$ (AC508-075-A, Thorlabs, Inc.), $f_4 = 175mm$ (32-884, Edmund Optics). The total magnification D of SHy-Cam can be estimated via the formula $M = \frac{f_2 \cdot f_4}{f_1 \cdot f_3} \approx 44x$. **B** Schematic of SPIM illumination and detetcion in relation to SHy-Cam. **C** Comparison of light sheet illumination area with the actual field of view of SHy-Cam image. The galvanometer scanner is tuned so that the generated scanning light sheet has the matching size in *y* direction with the SHy-Cam field of view to reduce unnecessary laser dose to the samples that is not being imaged.

Figure S2 Image pre-processing pipeline. **A** Manual cropping of the four channels from a brighfield image is performed in MATLAB (see Code Availability). Image shows the camera raw data with four channels distributed in the four quadrants of the sensor. **B** Screenshot of **'**SIN' channel here used as the reference channel. Green dots with numbers are the manually selected control points from the reference channel. Their corresponding control points from a registering moving channel (ASIN) shown in **C** are also manually selected. 8 control points pair on the hexagonal FOV edge and 1 control point pair in the center area of the FOV are selected. **D** Overlay image of 'SIN' and 'ASIN' channels before registration with visible misalignment. **E** Overlay image of 'SIN' and 'ASIN' after registration shows well aligned channels. **F** Screenshot of nine image tiles in Imaris Sticher 9.6 (Bitplane, Switzerland) before stitching. **G** Screenshot of stitched image after rotation and crop correction.

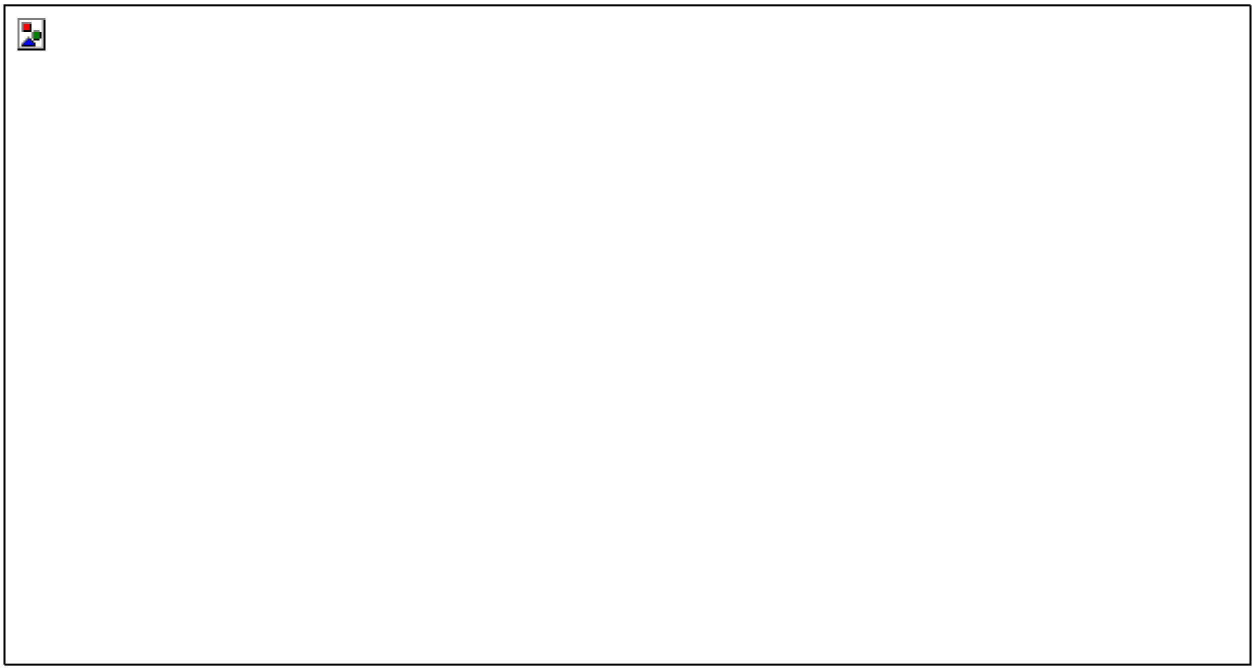

Figure S3 Spectral analysis on spectral phasor plane A The phasor plot of a pre-processed SHy-Cam image of a three-color *4dpf* transgenic zebrafish embryo Tg(krt4:lyn-EGFP;kdrl:mCherry;lyz:TagRFP) in HySP (see Code Availability). Three polygonal selections are applied to unmix three fluorescent signatures (Green: EGFP labeled superficial epithlial cells; Yellow: TagRFP labeled neutropils; Magenta: mCherry labeled vascularures). **B** Pseudo-color image in HySP representing, with saturated colors, the selections on phasor plane, color-matching the ROIs in **A**. The image is a lateral view of the trunk area of a zebrafish embryo, the slice shown is the 40th out of an 81-slice Z stack. **C** Maximum intensity projection image of the unmixed volume.

Channel \ Fluorophore	CFP	eGFP	eYFP	mCherry	iRFP670
ASIN	11%	12%	19%	35%	30%
ACOS	27%	31%	33%	19%	5%
SIN	29%	27%	20%	7%	12%
COS	12%	7%	6%	23%	37%
Total	79%	77%	78%	84%	84%

Table S1 SHy-Cam photon efficiency of 5 commonly used fluorescence. The photon efficiency estimates the percentage of the photons that are able to enter and exit SHy-Cam to the detector over the total emitted photons exciting the back aperture of the detection objective lens. The estimation is based on the light with 0° angle of incidence (AOI) for simplicity reasons. The photon loss along the optical train of SHy-Cam is calculated by taking the loss of each optical surface between the first surface of the filter pair F and the last surface of the tube lenses TL_{1-4} (see Figure S1). For achromatic doublet lenses, the first and last surface photon loss is estimated by the average reflection value of the anti-reflection coatings within 400nm-700nm (Edmund Optics: <1.75%, Thorlabs< 0.5%). The glass-to-glass photon loss by reflection is estimated by Fresnel equation under 0° AOI condition between two glasses with different refractive indices. Photon loss at folding mirrors and beamsplitter is estimated at 1% per the coating specification from Thorlabs. The transmittance and reflectance profiles of two custom sinusoidal dichroic mirrors were provided by manufacturer.