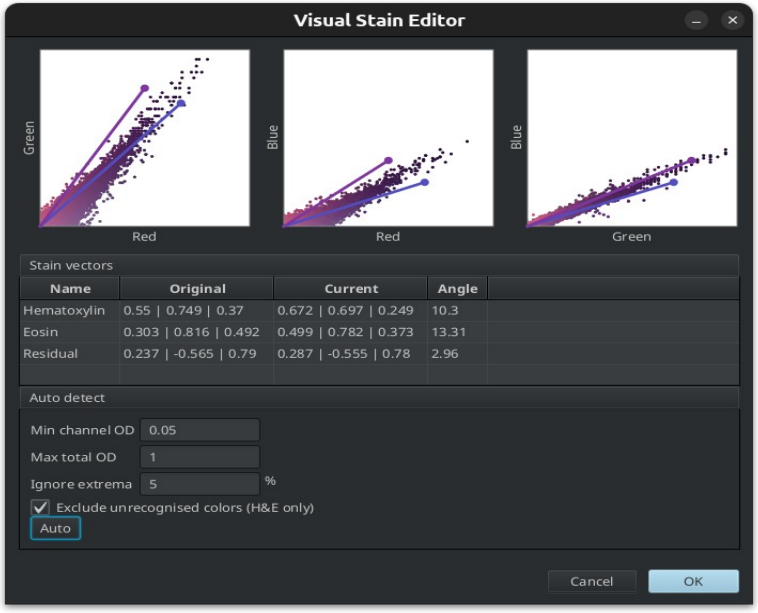
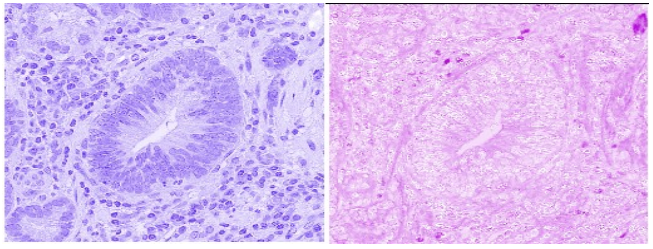
Chapter 3

Stain Separation by Color Deconvolution

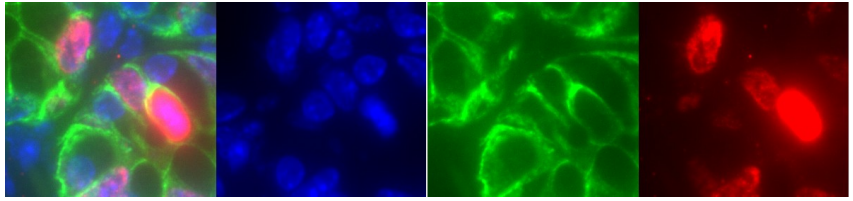




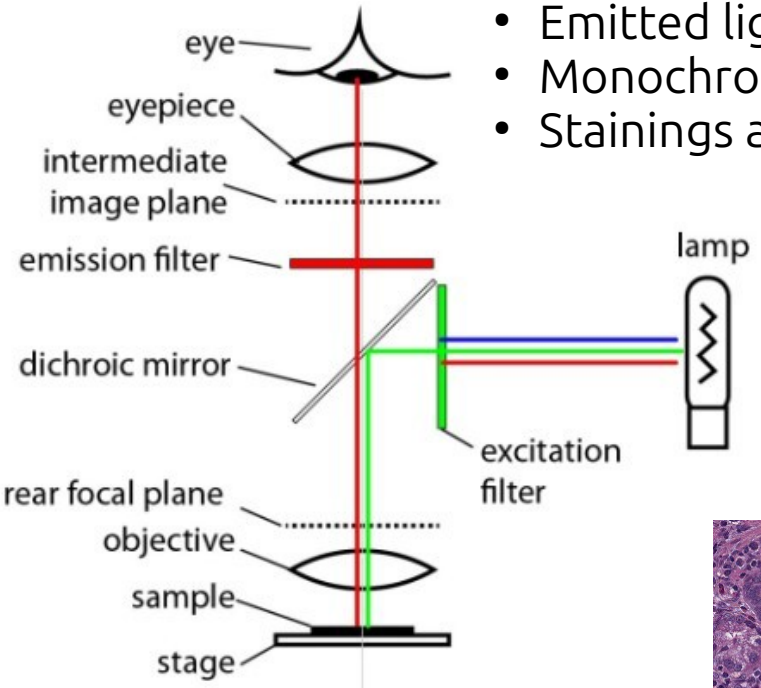
Hematoxylin Eosin

BAECKER Volker
BENEDETTI Clément
HASSEN-KHODJA Cédric

Fluorescence vs. Brightfield

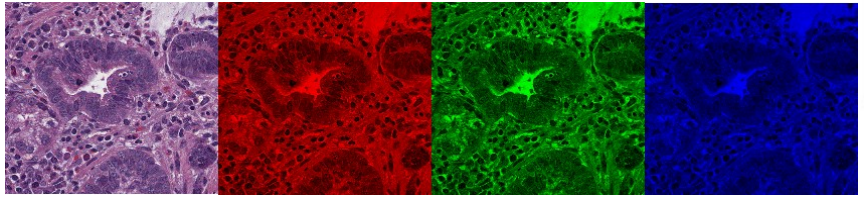


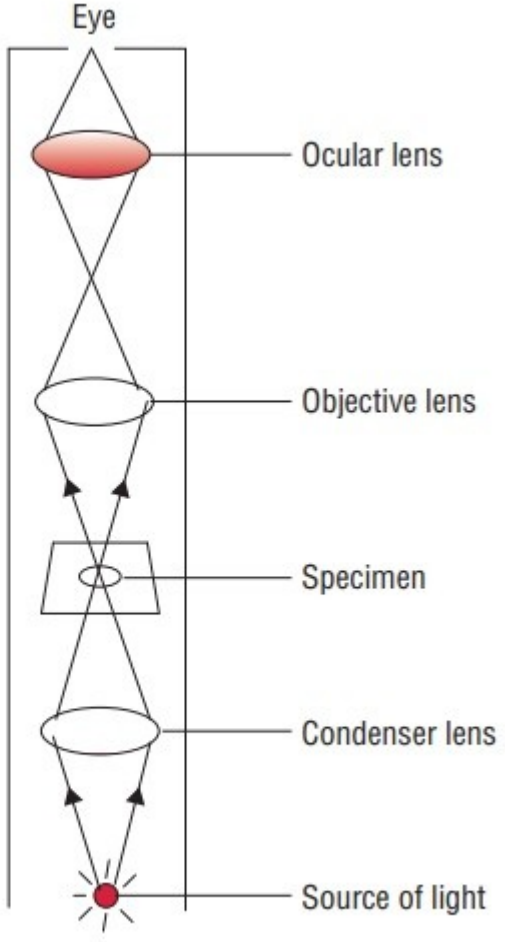
- Emitted light
- Monochrome camera
- Stainings acquired individually



The epi-fluorescence microscope[2]

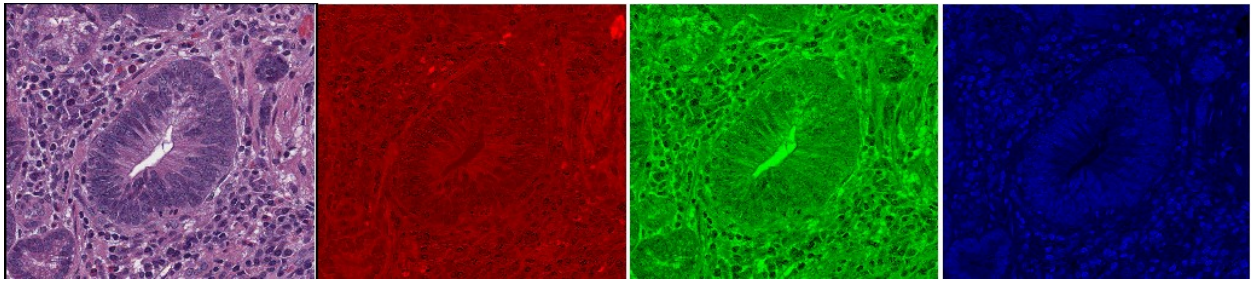
- Transmitted light (light not absorbed)
- RGB camera
- Stainings acquired together





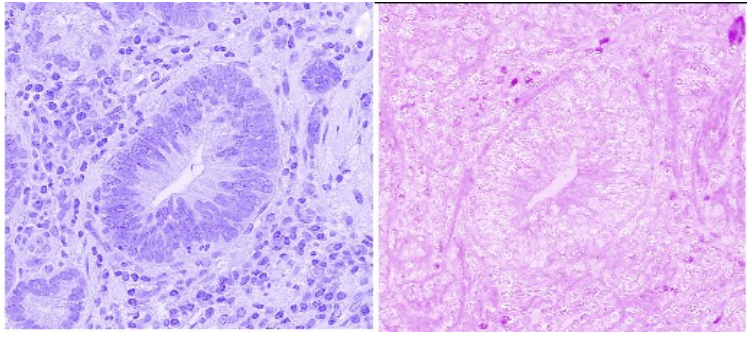
The compound light microscope [1]

Color Deconvolution



Objective:

- calculate the contribution of each of the stains,
- based on the stain-specific RGB absorption.



Hematoxylin Eosin

Ruifrok, A C, and D A Johnston. Quantification of Histochemical Staining by Color Deconvolution. Analytical and Quantitative Cytology and Histology, 23 (4): 291–9, 2001.

Lambert-Beer's law

The transmitted light I_C in a channel c (red, green, blue) depends on

- The entering light intensity in the channel $I_{0,C}$
- The amount of stain A
- The absorption factor of the stain in the channel c_C

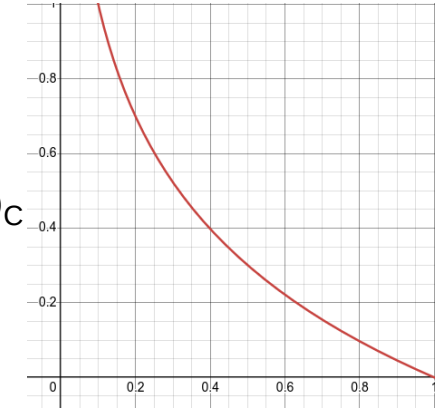
$$I_C = I_{0,C} \cdot e^{-A \cdot c_C}$$

The relation between the intensity in the image and the amount of stain is not linear!

But the optical density is linear in the amount of stain for each channel

$$OD_C = -\log_{10}\left(\frac{I_C}{I_{0,C}}\right) = A \cdot c_C$$

OD_C



OD-matrix

Each pure stain has a specific OD-RGB vector

	R	G	B
Hematoxylin	0.18	0.20	0.08
Eosin	0.01	0.13	0.01
DAB	0.10	0.21	0.29

	R	G	B
Hematoxylin	p_{11}	p_{21}	p_{31}
Eosin	p_{12}	p_{22}	p_{32}
DAB	p_{13}	p_{23}	p_{33}

To separate stains

- Normalize and inverse OD matrix
- Multiply with OD-image

Color Deconvolution in QuPath

We need the entering light intensity for each channel

- We assume it to be the background value
- i.e. the value in an empty region

We need to provide the OD-RGB vecors for the pure stains

3 possibilities

- Predefined values
- From locations of pure stains in the image
- Auto-estimate from RGB distribution

Color Deconvolution - Predefined Values

Predefined values are used, when the image type is set to

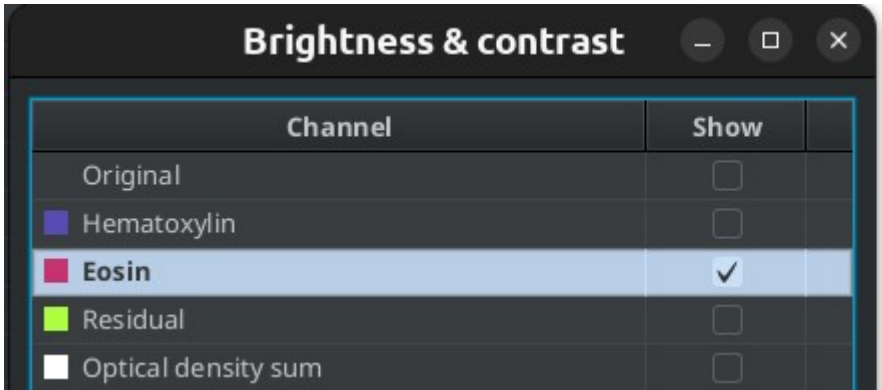
- Brightfield H&E
- Brightfield H-Dab
- Brightfield other

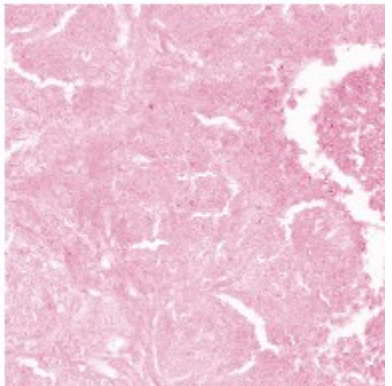
You can see and modify the values on the image pane

Image type	Brightfield (H&E)
Stain 1	Hematoxylin: 0.651 0.701 0.29
Stain 2	Eosin: 0.216 0.801 0.558
Stain 3	Residual: 0.316 -0.598 0.737
Background	255 255 255

You can check the result

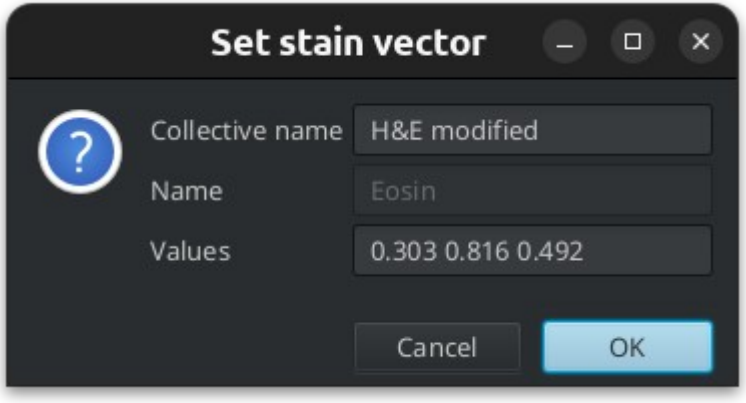
- Via the B&C tool
- The channel-viewer

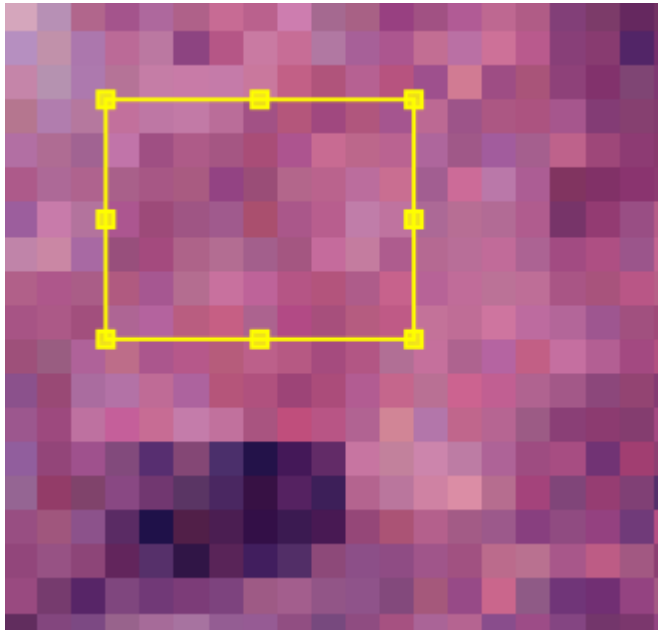




Stain OD-RGB values from ROIs

Image type	Brightfield (H&E)
Stain 1	Hematoxylin: 0.55 0.749 0.37
Stain 2	Eosin: 0.216 0.801 0.558
Stain 3	Residual: 0.32 -0.598 0.735
Background	255 255 255



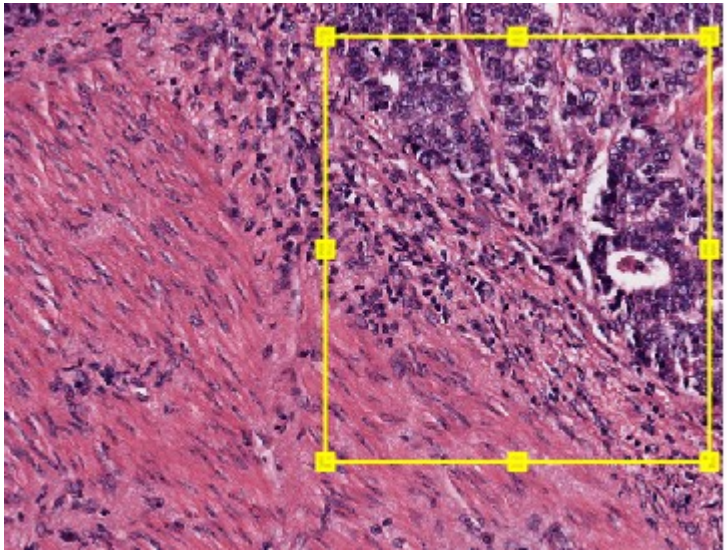


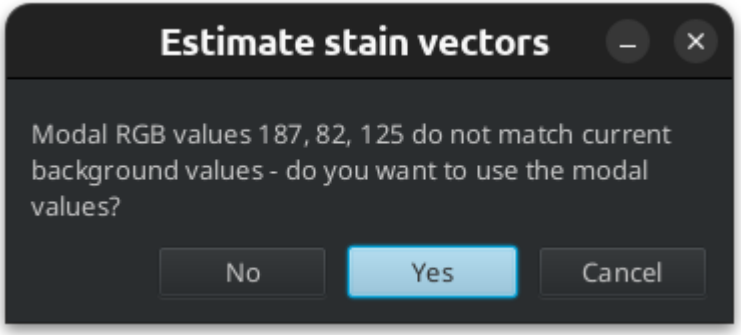
Estimate stain vectors

Make an annotation that contains

- Background
- pure stain samples for each stain

Run Analyze>Estimate stain vectors



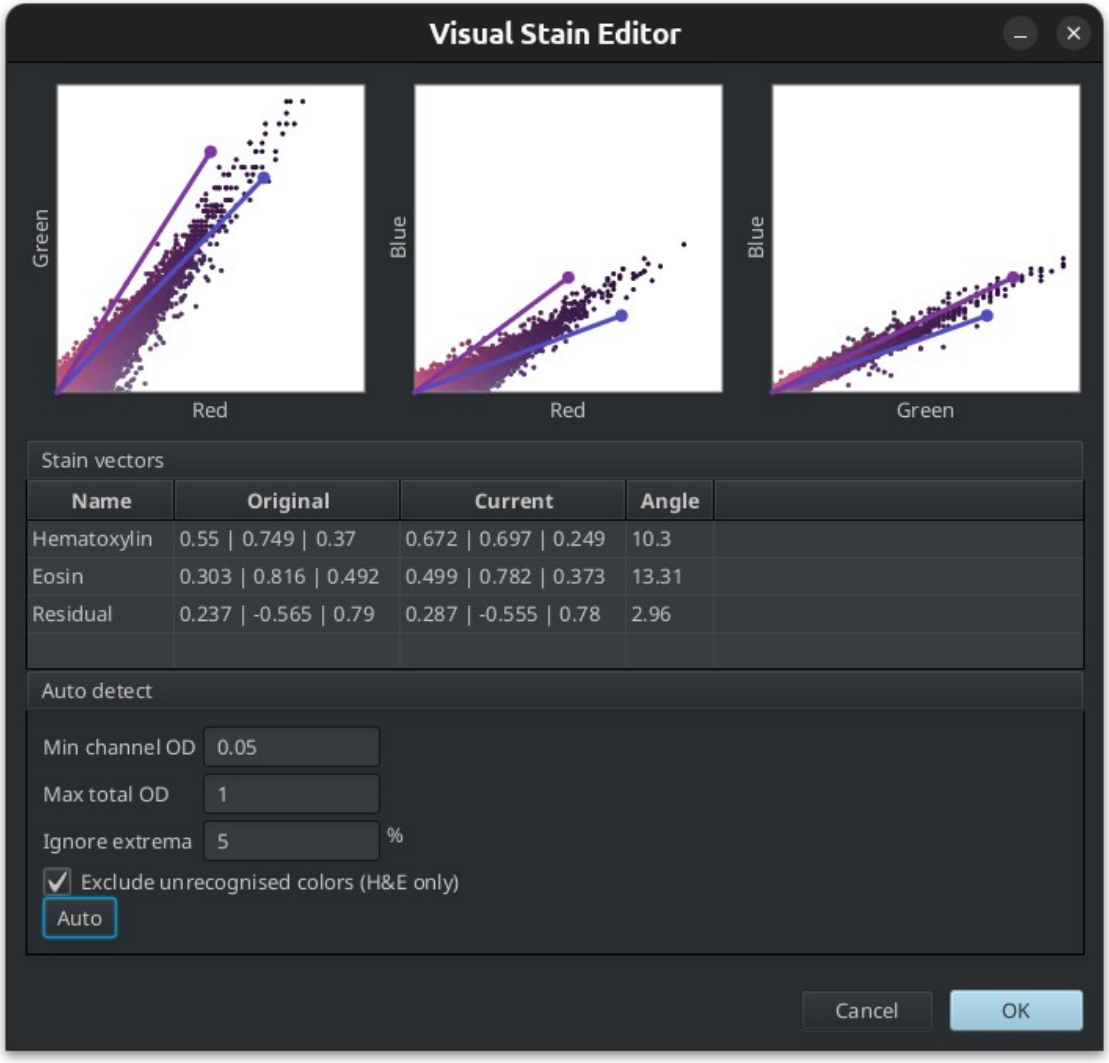


Estimate stain vectors

Move vectors or

Use auto detect

- Parameters
  - Min. channel OD
  - Max total OD
  - Ignore extrema

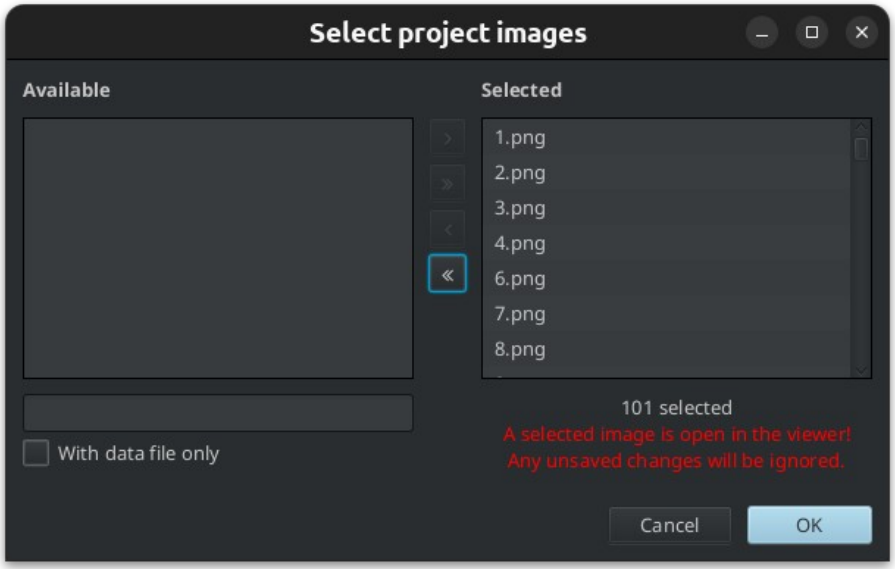


Macenko, M., Niethammer, M., Marron, J.S., Borland, D., Woosley, J.T., Xiaojun Guan, Schmitt, C., Thomas, N.E., 2009. **A method for normalizing histology slides for quantitative analysis,** in: 2009 IEEE International Symposium on Biomedical Imaging: From Nano to Macro. Presented at the 2009 IEEE International Symposium on Biomedical Imaging: From Nano to Macro (ISBI), IEEE, Boston, MA, USA, pp. 1107–1110. https://doi.org/10.1109/ISBI.2009.5193250

Set stain vectors for all images in the project

Set for one image, then:

- Automate>Create command history script
- Remove unwanted commands
- Run>Run for project



Script Editor

File Edit View Language Insert Run Help

Scripts
*Untitled 1
*Untitled 2
*Untitled 3

```
setImageType('BRIGHTFIELD_H_E');
setColorDeconvolutionStains('{"Name" : "H&E modified",
                             "Stain 1" : "Hematoxylin", "Values 1" : "0.6737 0.69433 0.25304",
                             "Stain 2" : "Eosin", "Values 2" : "0.43378 0.7654 0.47539",
                             "Background" : " 195 93 136"}');
```

Limits

Color deconvolution

- 2 or 3 stains
- Helpful to detect cells / structures
- Positive cells

Color deconvolution

- Can not be interpreted quantitatively
- Especially DAB
- DAB
  - Scatters light rather than absorbing
  - Does not follow the Beer-Lambert law

Images

[1] The compound light microscope from https://www.brainkart.com/article/Light-microscopy_17818/

[2] The epi-fluorescence microscope from https://bookdown.org/jcog196013/BS2010_Notes/wide-field-microscopy.html