光學顯微鏡
基本原理及運用

錢宗良
臺灣大學醫學院
解剖學暨細胞生物學研究所
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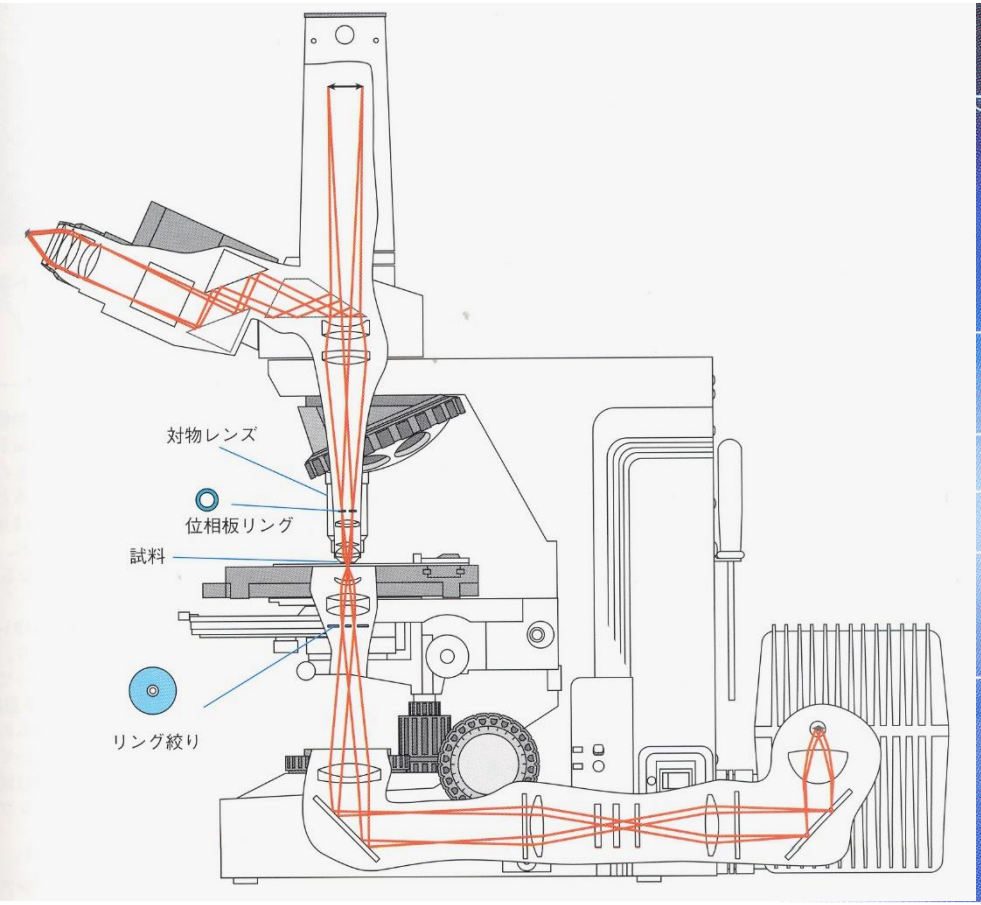
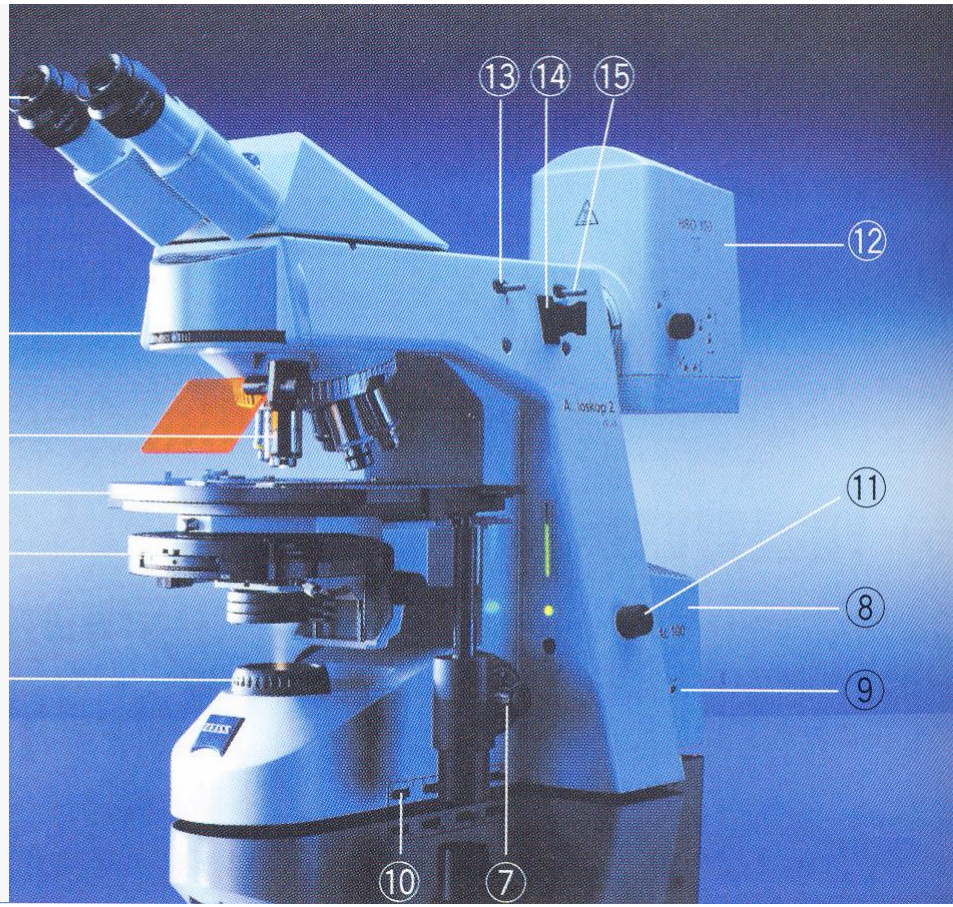
光學顯微鏡





顯微鏡的分類

正立顯微鏡：觀察組織切片

倒立顯微鏡：觀察培養活細胞

實體顯微鏡：解剖及立體定位

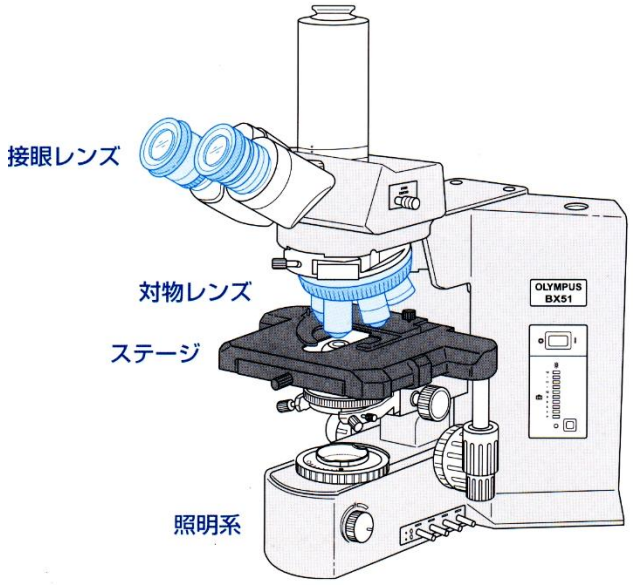


図1　正立型顕微鏡

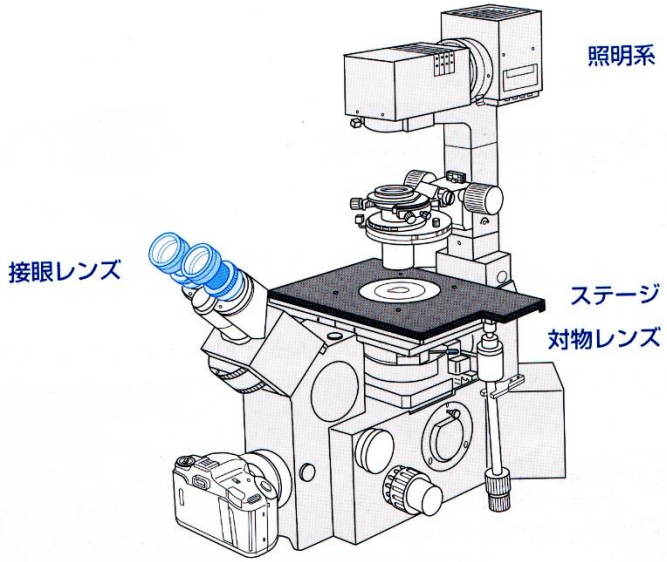
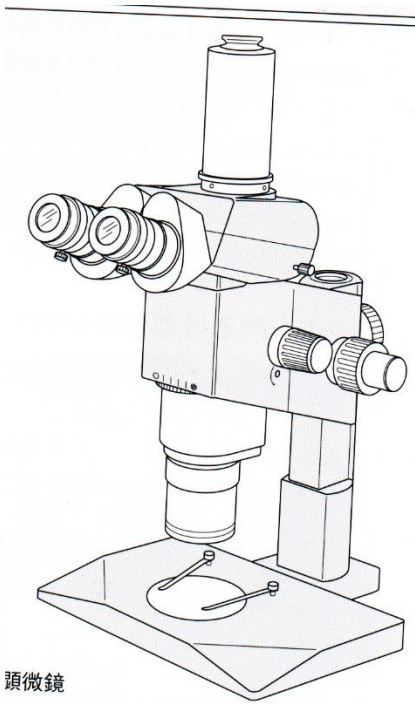


図2　倒立型顕微鏡



顕微鏡

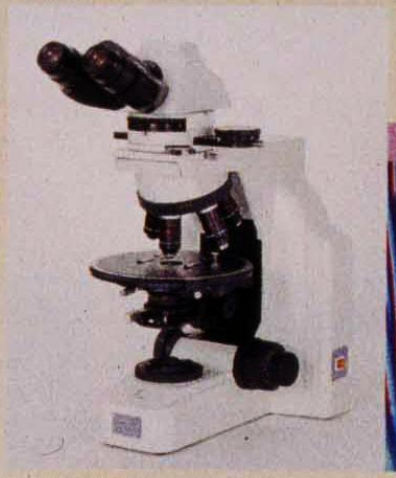
D いろいろな顕微鏡と装置

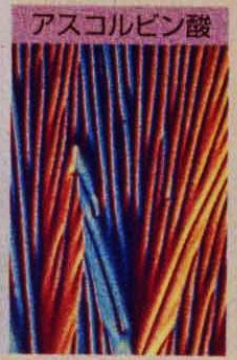
試料をいろいろな面から観察できるような光学顕微鏡と付属装置が開発されている。

●微分干渉顕微鏡

細胞分裂・原形質流動・アメーバ運動・繊毛運動の観察に使用。

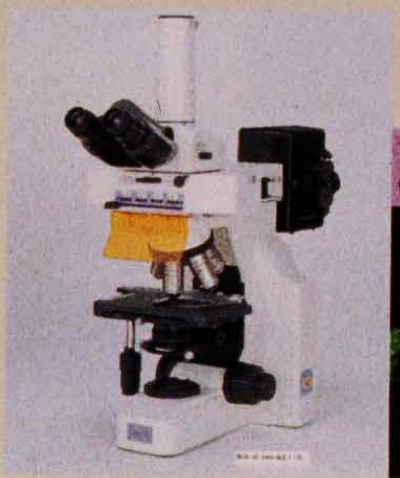
●偏光顕微鏡

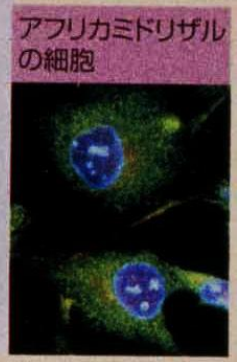




偏光板を利用した顕微鏡で，筋繊維などの微細構造の観察に使用。

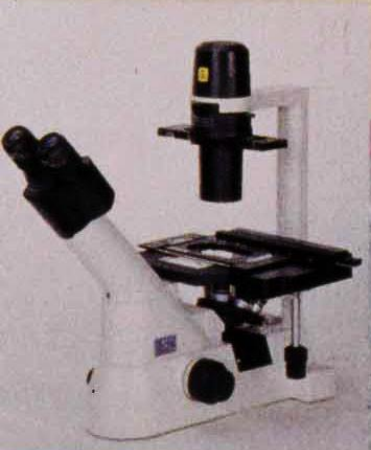
●蛍光顕微鏡





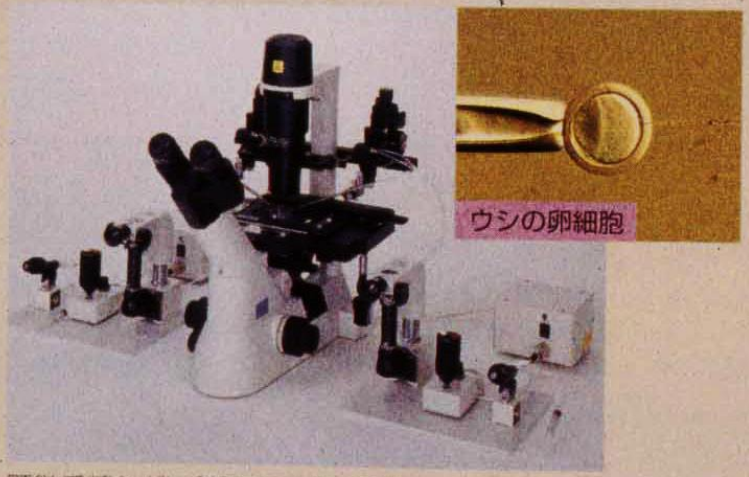
蛍光色素で抗体などを着色し，試料の発する蛍光を観察する。

●培養倒立顕微鏡



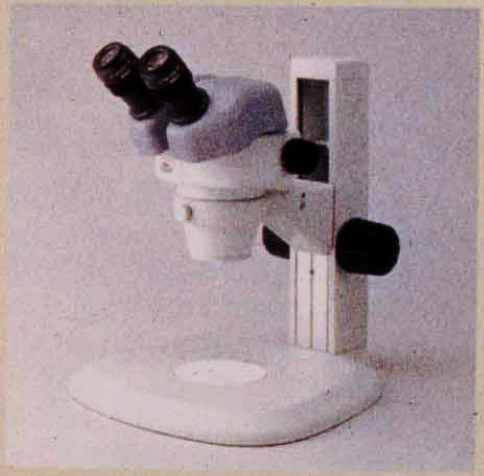
培養容器に入った細胞などの観察に使う。

●マイクロマニピュレーター



顕微受精などに利用する付属装置。

●実体顕微鏡

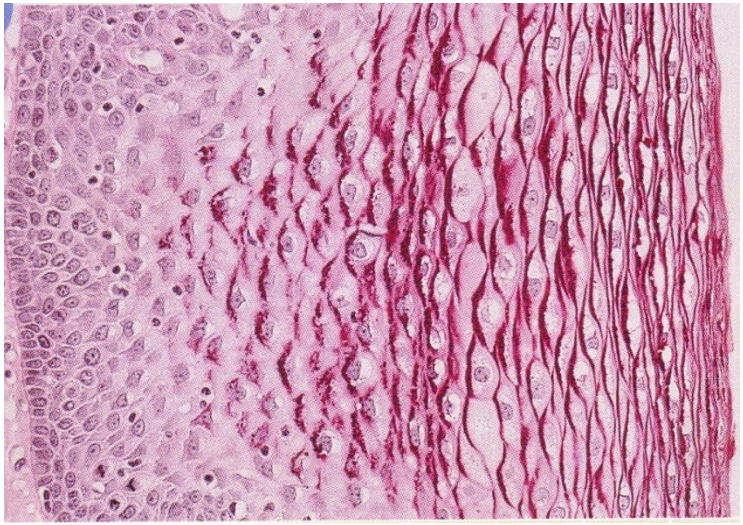


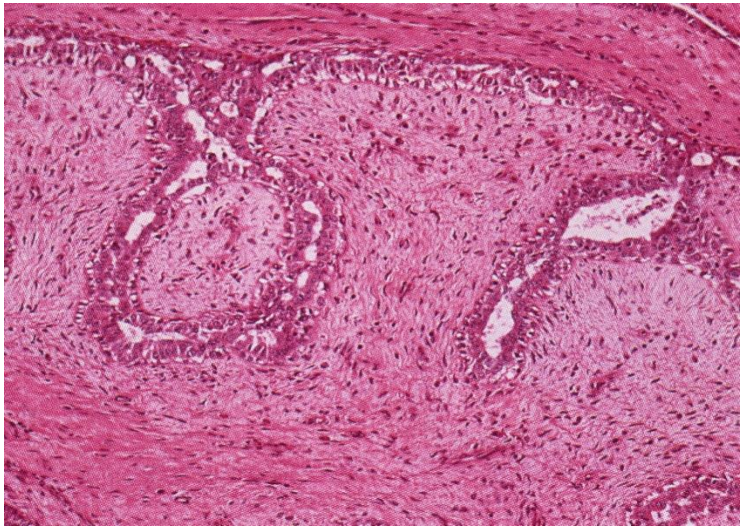
生試料を正立像で立体的に観察できる。

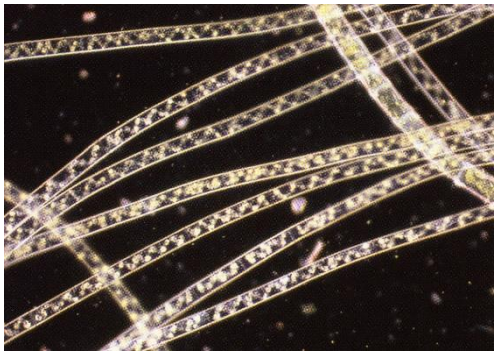
觀察法的顯微鏡分類

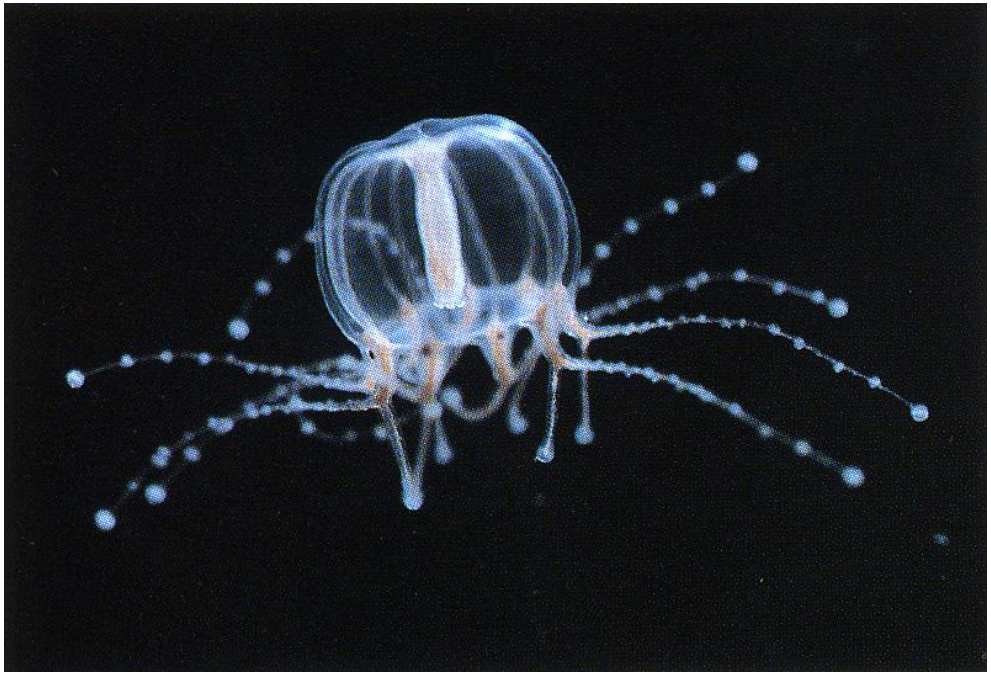
明視野 (bright field) ：一般光學組織切片

暗視野 (dark field) ：小動物如線蟲及纖毛鞭毛結構

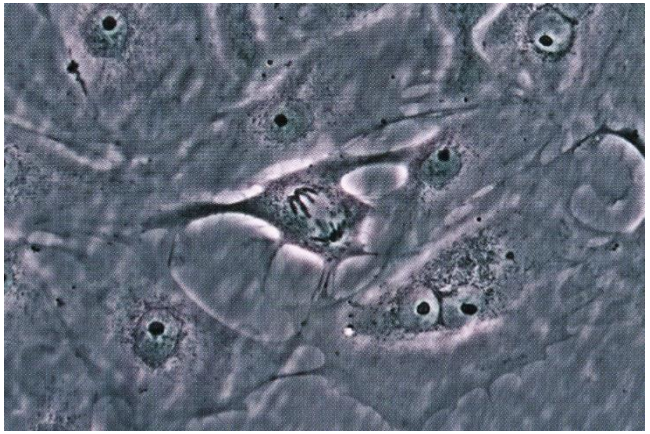
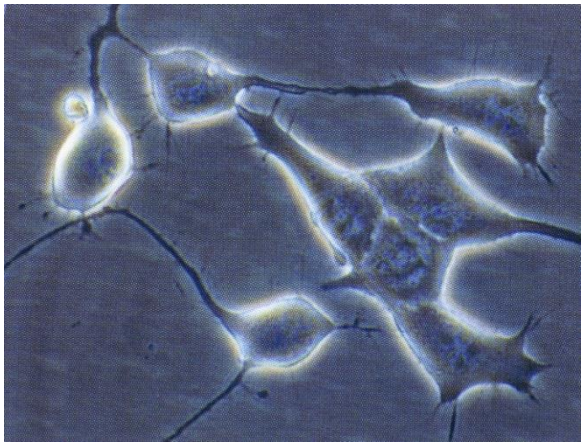
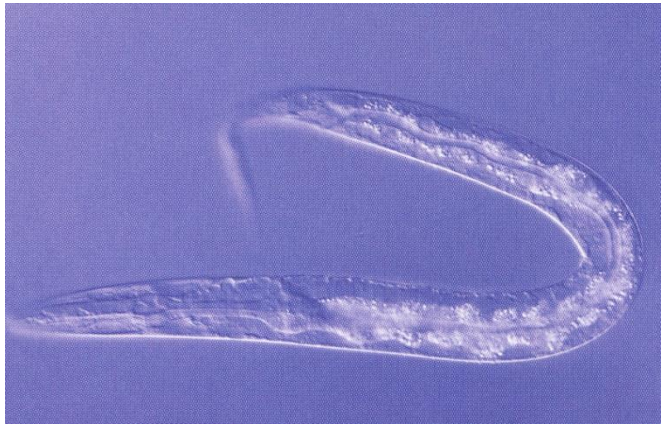
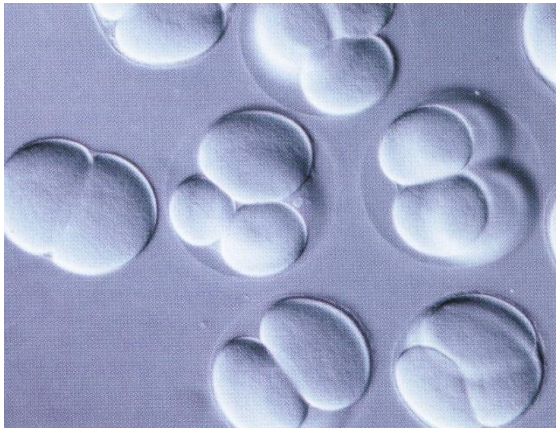
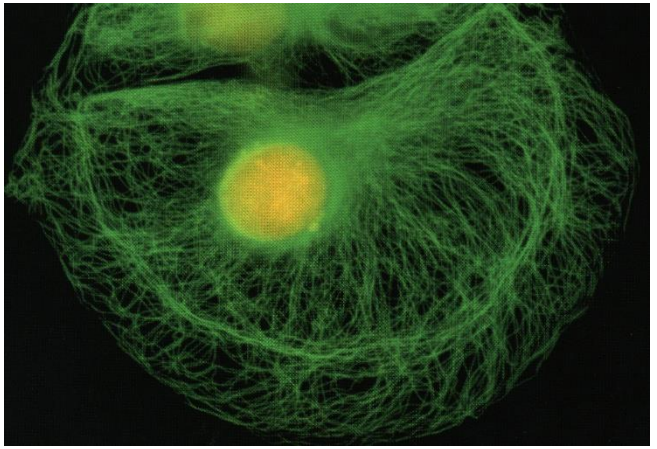








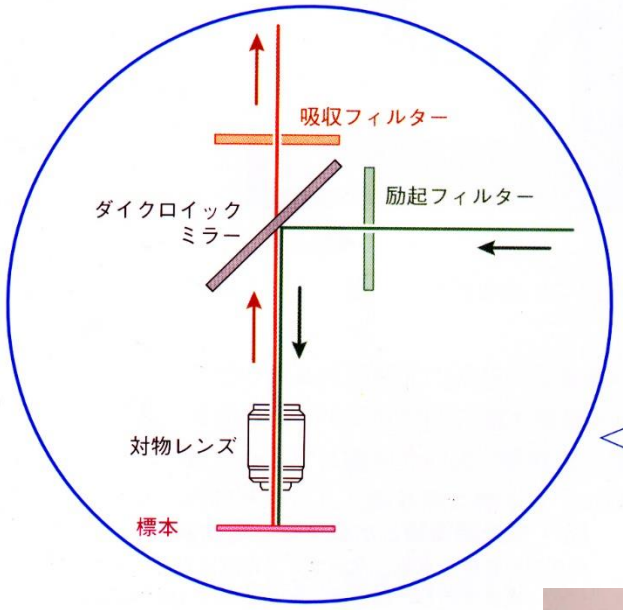
偏光 (polarized light) ：堅硬的結晶結構等
位相差 (phase contrast) ：一般培養活細胞
微分干涉 (differential interference contrast, DIC) ：活體卵細胞及線蟲等
螢光 (fluorescence) ：一般螢光染色標示或活體螢光如GFP等

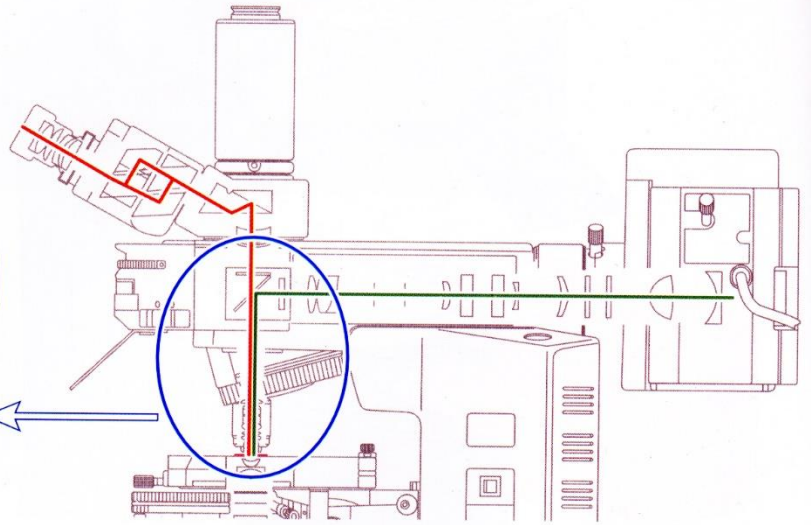







照明光源路徑:

穿透光照明：一般光學組織切片，薄具透光性；對比與染色

反射照明：解剖及實體顯微鏡，螢光顯微鏡；表面光源反射





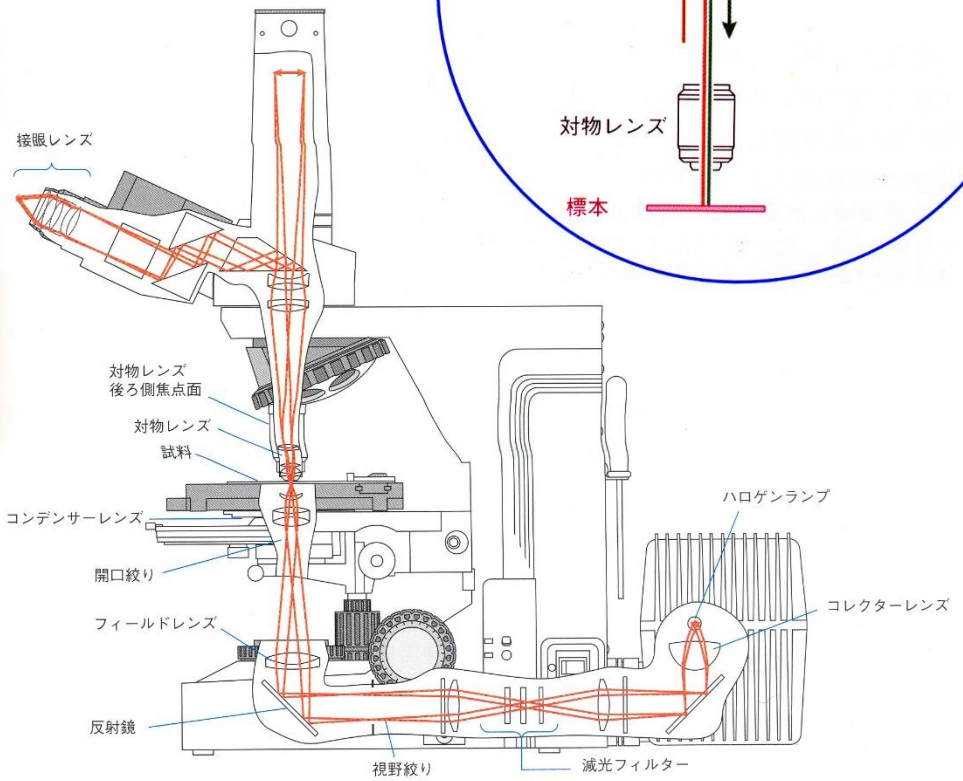
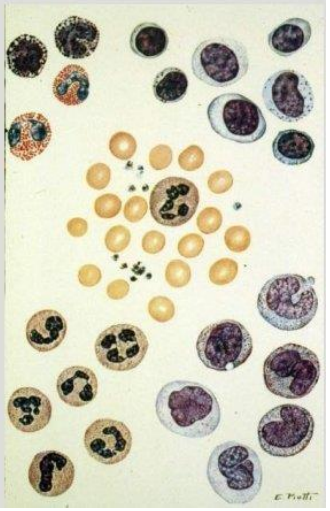


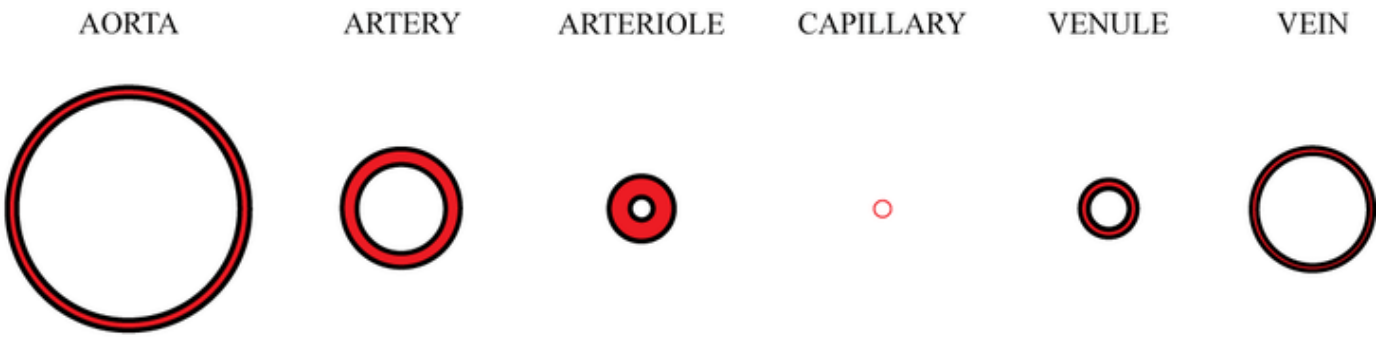
TABLE 1.3. Eye versus Instrument Resolution

	DISTANCE BETWEEN RESOLVABLE POINTS
Human eye	0.2 mm
Bright-field microscope	0.2 μm
SEM	2.5 nm
TEM	
Theoretical	0.05 nm
Tissue section	1.0 nm

SIZE OF HUMAN BLOOD CELLS

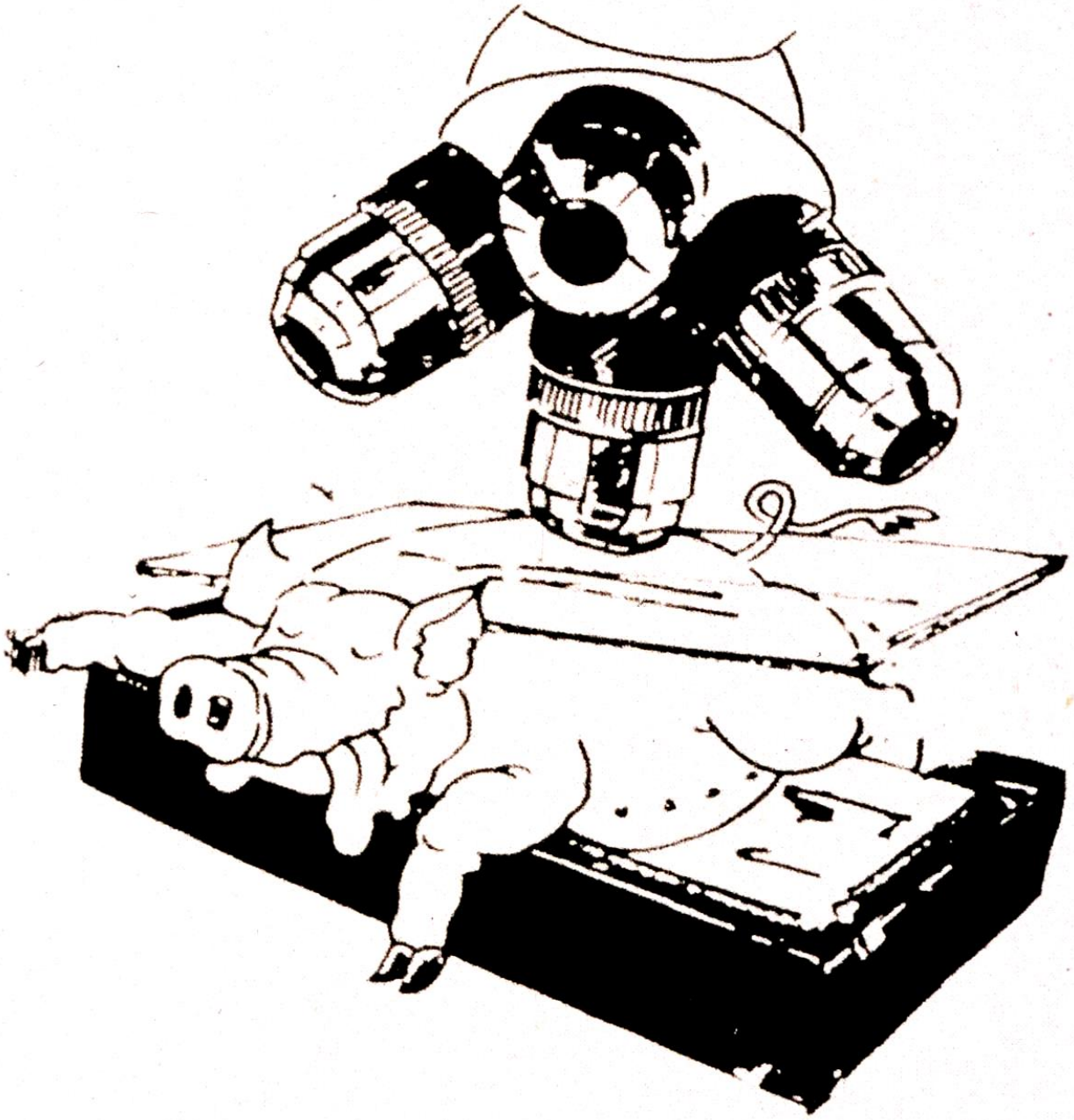
CELL/PLATELET	SIZE
ERYTHROCYTES	6.5-8 μm
LEUKOCYTES (WBC)	
% of WBC	
NEUTROPHIL	12-15 μm
60-70%	
LYMPHOCYTE	6-18 μm
25%	
MONOCYTE	12-20 μm
5%	
EOSINOPHIL	12-15 μm
-4%	
BASOPHIL	12-15 μm
-1%	
PLATELETS	2-4 μm





	AORTA	ARTERY	ARTERIOLE	CAPILLARY	VENULE	VEIN
Human	25,000/2,000	4,000/1,000	30/20	8/1	20/2	5,000/500
Mouse	535/55	150/50	18/4.7	4/0.3	14/1	250/50

The typical diameters/wall thicknesses of blood vessel for humans and mice are given in micrometers. The smallest capillaries have diameters of a very few micrometers and a wall thickness of about one micrometer (**μm**).



(図：カールツァイス社)

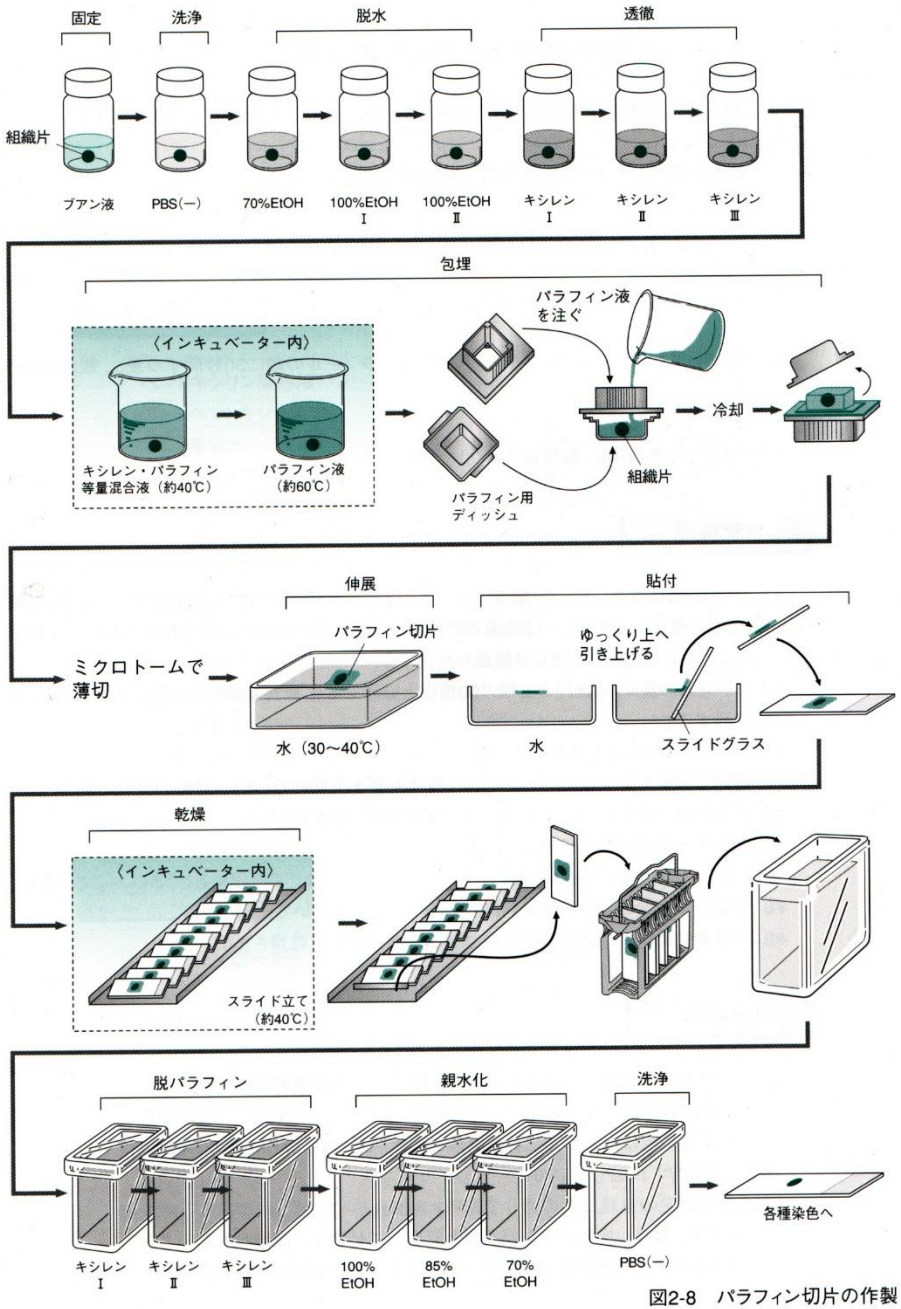
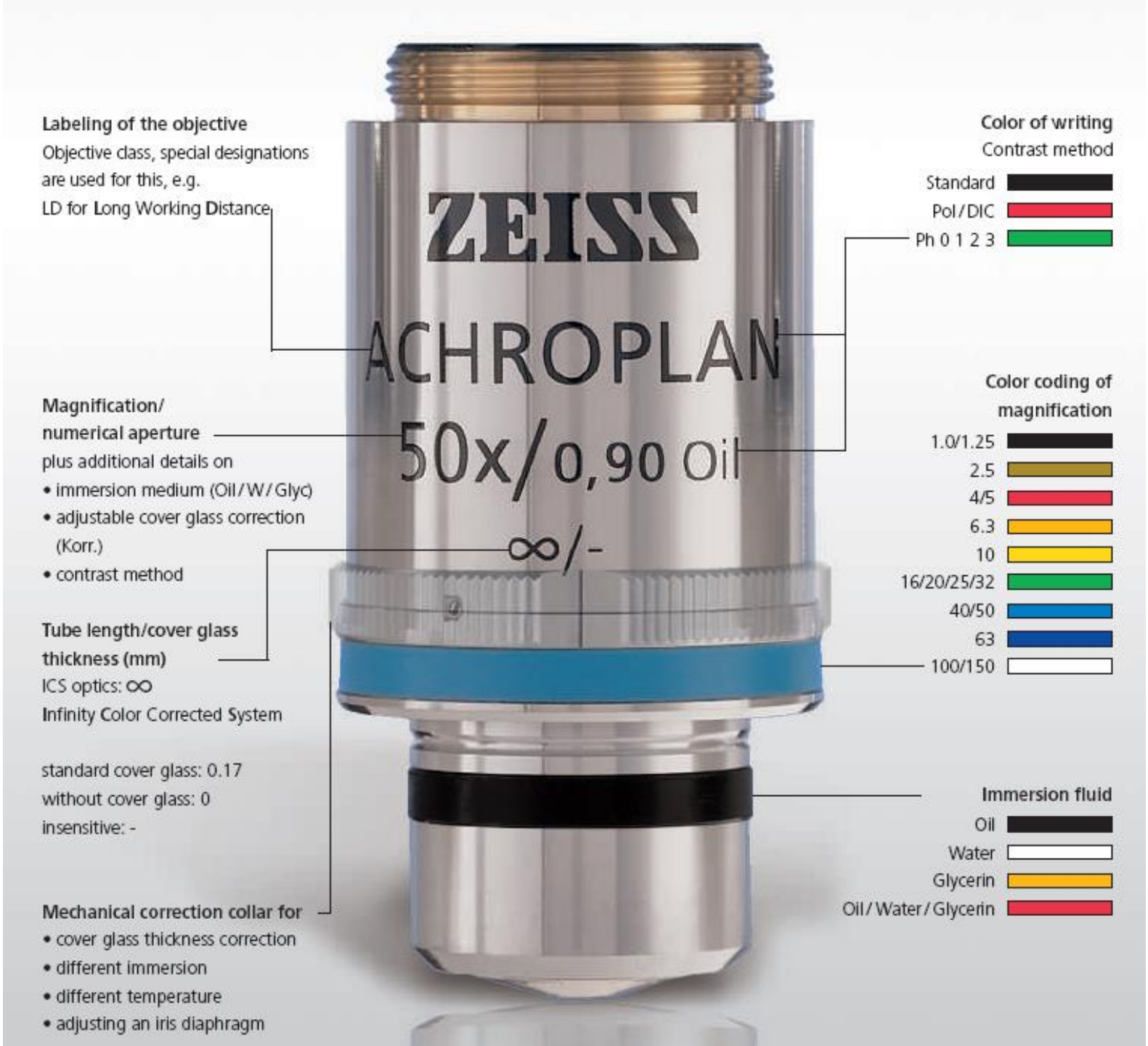


図2-8　パラフィン切片の作製

Cryostat sectioning

物鏡

- 開口值 /NA
- 消色差
- 工作距離
- 色帶
- 蓋玻片厚度



Labeling of the objective
Objective class, special designations are used for this, e.g.
LD for **L**ong Working **D**istance

Magnification/ numerical aperture
plus additional details on
- immersion medium (Oil/W/Glyc)
- adjustable cover glass correction (Korr.)
- contrast method

Tube length/cover glass thickness (mm)
ICS optics: ∞
Infinity **C**olor Corrected **S**ystem

standard cover glass: 0.17
without cover glass: 0
insensitive: -

Mechanical correction collar for
- cover glass thickness correction
- different immersion
- different temperature
- adjusting an iris diaphragm

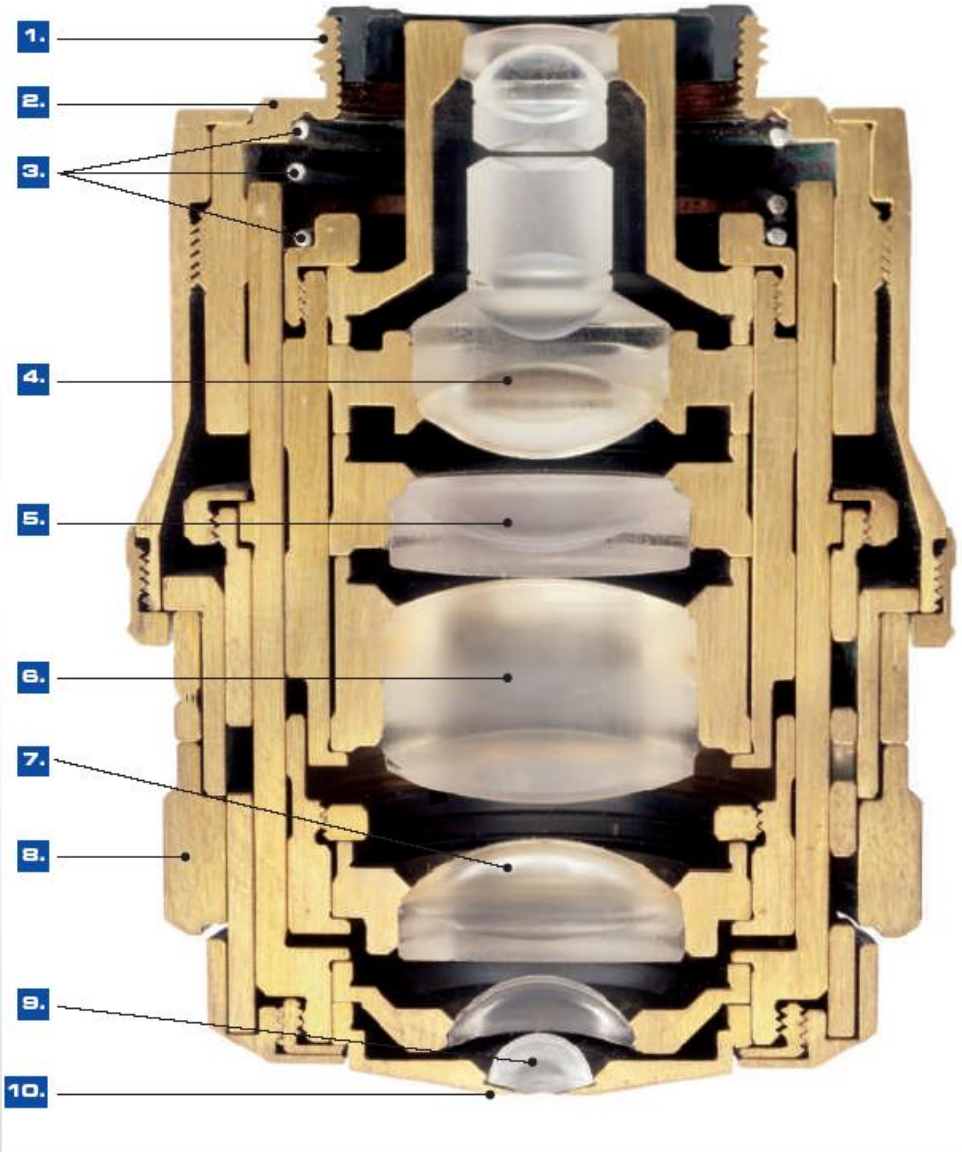
Color of writing
Contrast method
Standard
Pol/DIC
Ph 0 1 2 3

Color coding of magnification
1.0/1.25
2.5
4/5
6.3
10
16/20/25/32
40/50
63
100/150

Immersion fluid
Oil
Water
Glycerin
Oil/Water/Glycerin

Lens:

Most important part of Microscope

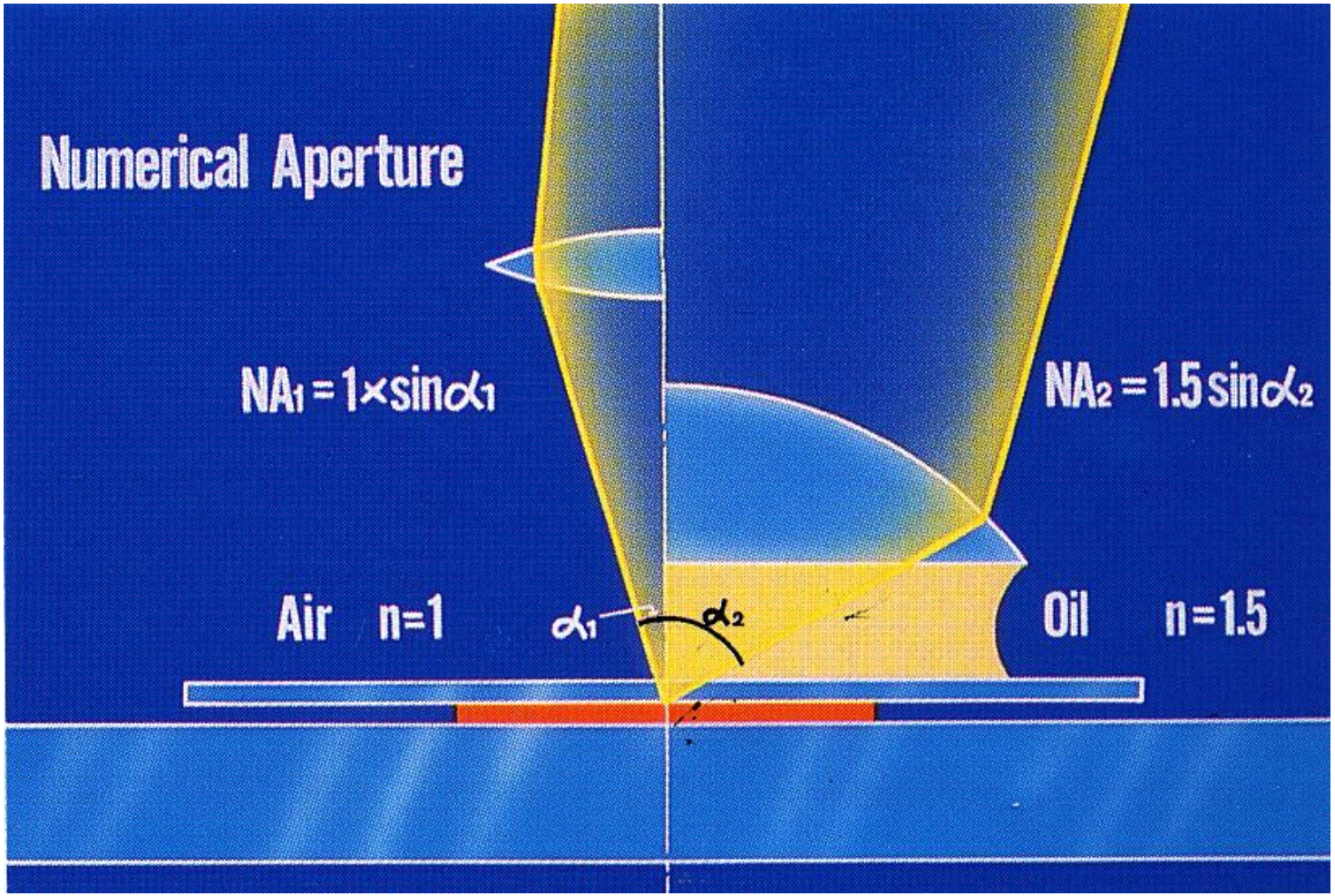


Cross section of an objective

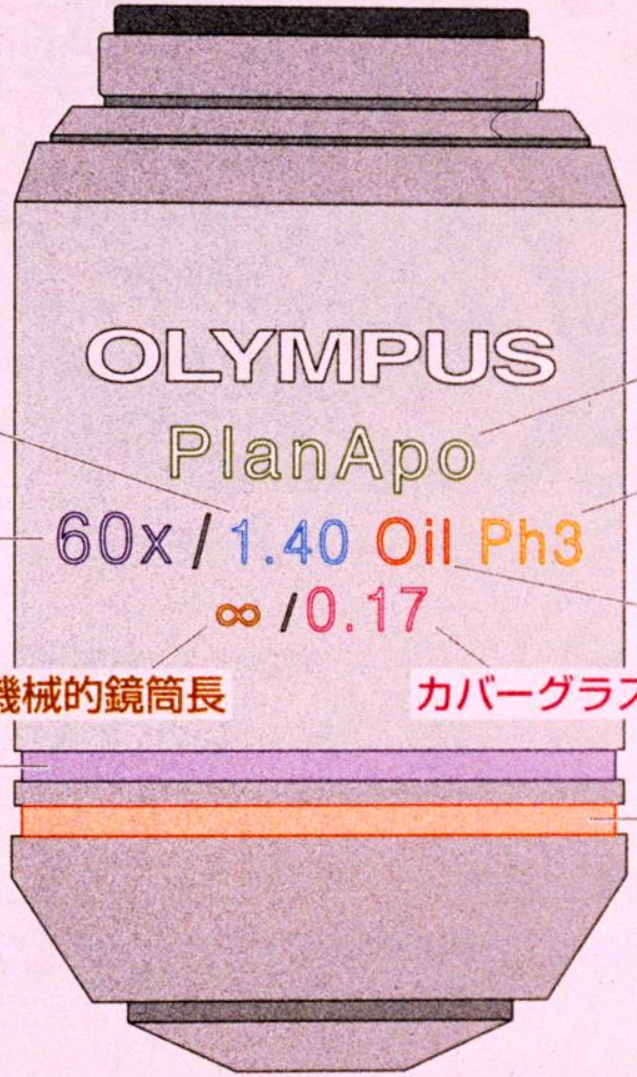
1 Objective thread
2 Stop face of the objective
3. Spring system for the specimen-protection mechanism
4 - 7. Lens groups for the correction of image errors
8. Correction collar for adapting to deviating cover glass thicknesses or temperatures
9. Front lens system
10. Front lens holder

開口值 (NA)

- 開口值越大，鏡頭聚光能力越強，解析能力越強．



A



開口数

倍率

種類

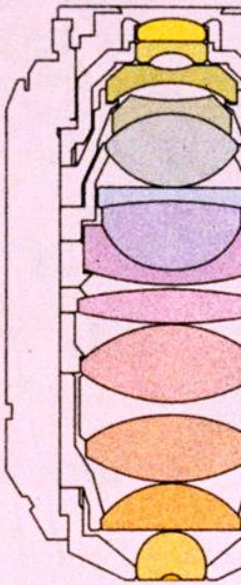
対応するリングスリット
位相差観察用対物レンズにのみ表示がある

浸液
Oil　イマージョンオイル
W　水
Gly　グリセリン

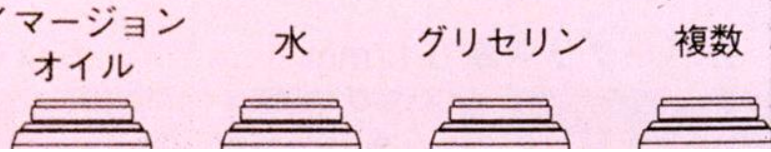
機械的鏡筒長

カバーグラス厚

B



浸液表示色帯



- **Correction efficiency (UV – VIS – IR)**
- **N. A.**
- **Type (What's CS Objectives ?) - Quality**

  PL-APO (Delta, HCS)

  PL-Fluotar

  N Plan

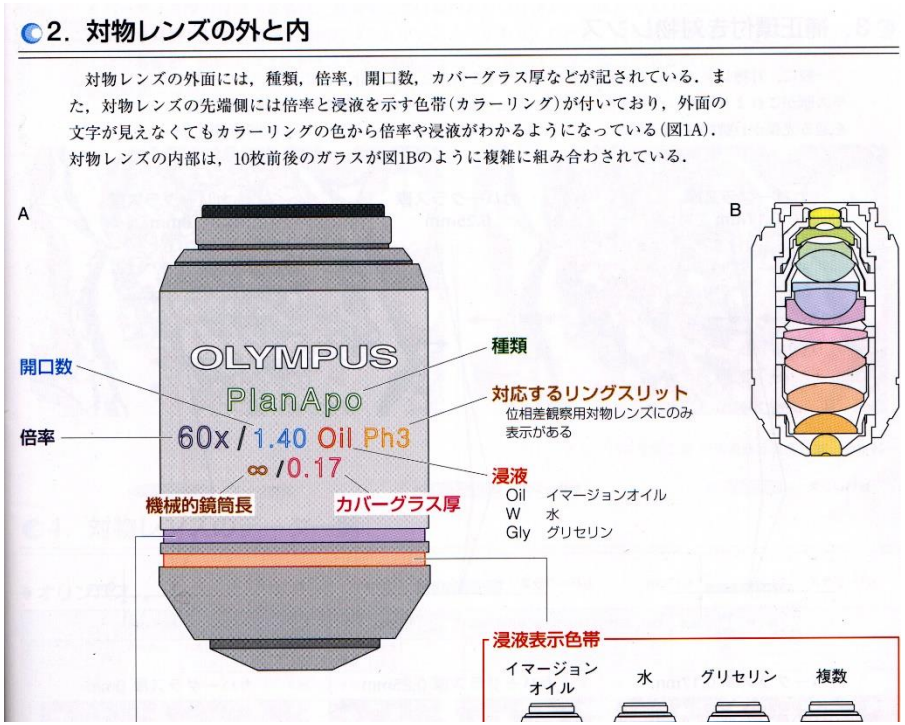
  C Plan

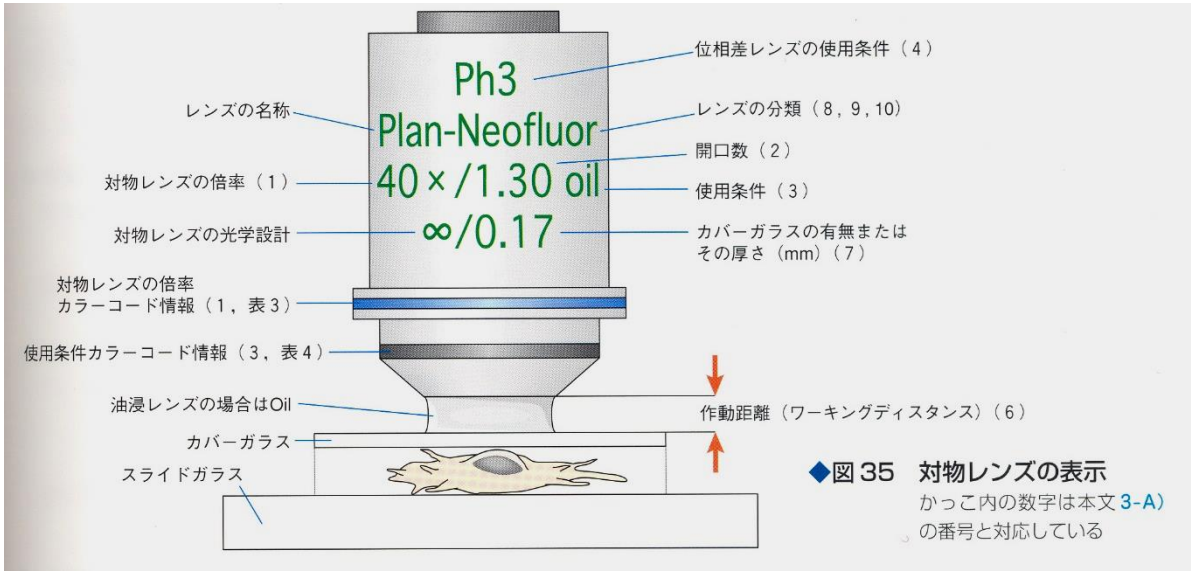
  Air, Water, Oil (Imm)

  Cover slip thickness

  correction ?

  Chamber Type ?

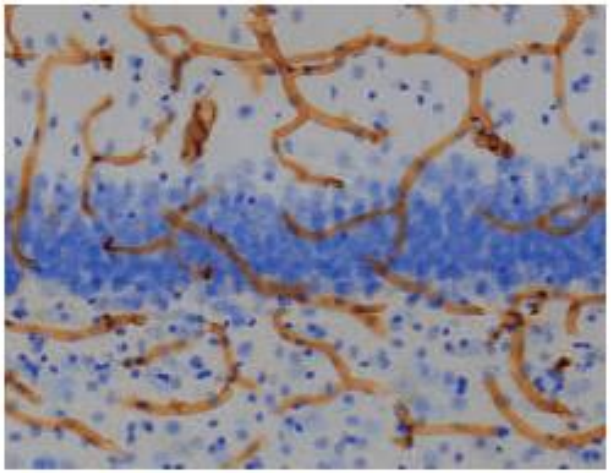




The Best Lens: Apochromat

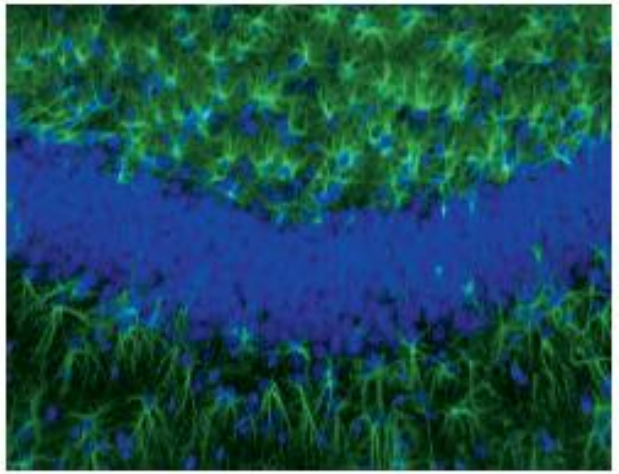
Plan-APOCHROMAT –
the precision performers

With the best color correction and highest numerical apertures, Plan-APOCHROMAT objectives deliver brilliant images in brightfield, DIC and fluorescence techniques. Their outstanding point spread function and extreme chromatic correction are particularly impressive. High resolution and excellent image sharpness make even the finest details and color nuances visible.



C-APOCHROMAT – the top performers

These high-performance objectives are able to compensate optically for different refractive indices and layer thicknesses of the mounting medium by means of a correction collar. They are perfectly suited to extremely demanding applications in research of living organisms and immersion specimens. For brilliant images in all applications and 3D techniques such as confocal Laser Scanning Microscopy, ApoTome and 3D Deconvolution.



The Best Lens: Apochromat

Resolution table using green light with $\lambda = 0.550$ µm:

Magnification	/	NA	Resolution (µm)
10x	/	0.30	1.10
40x	/	0.75	0.45
63x	/	1.40 Oil	0.24
100x	/	1.30 Oil	0.26

Plan-APOCHROMAT: protects sensitive samples

Plan-APOCHROMAT objectives demonstrate top-class optical performance. They make it possible to see structures at the boundary of what is visible. Their outstanding performance features include: excellent correction, extremely high apertures and maximum resolution, color purity, contrast and flatness of field. All this combines to produce brilliant, needle-sharp images for observation, digital documentation and, in particular, fluorescence applications. The i Plan-APOCHROMAT of the 63x objective has been developed specifically for Live Cell Imaging – for optimal focus stability for time-lapse experiments.

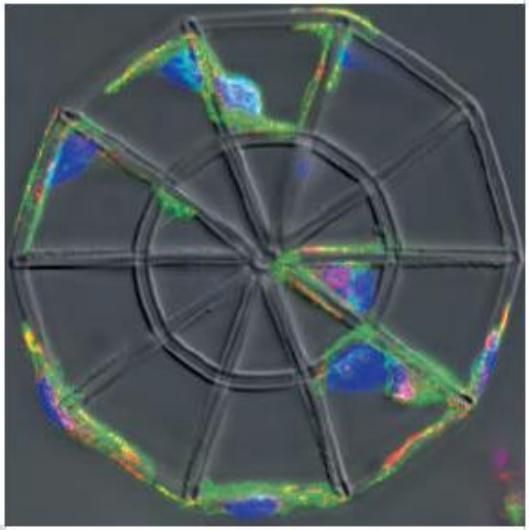


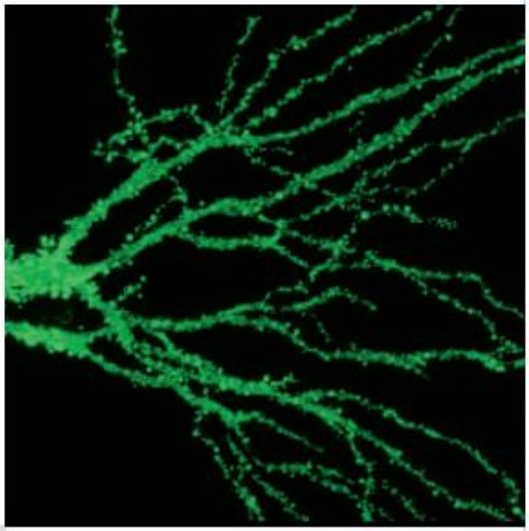
Image courtesy of Martin Bastmeyer und Franziska Klein, University of Karlsruhe, Germany

- Field of view: 25 mm
- Flatness: ★★★★★
- Color correction: ★★★★★

Objectives with optimum correction of flatness of field and color; suitable for Digital Imaging

W Plan-APOCHROMAT: apochromatically correct

The immersion variant of the Plan-APOCHROMAT series – an addition to the water objectives of the ACHROPLAN class – has been specifically designed for electrophysiology. W Plan-APOCHROMAT objectives have apochromatic correction from visible light to the near infrared (VIS - IR) and are intended for use without a cover glass. Typical transmission values are greater than 80% from 450 nm to 1,000 nm and greater than 50% at 365 nm. These are also ideal prerequisites for use in 2-photon microscopy. The front of this slender objective is made of a special inert plastic that was originally developed for food technology.

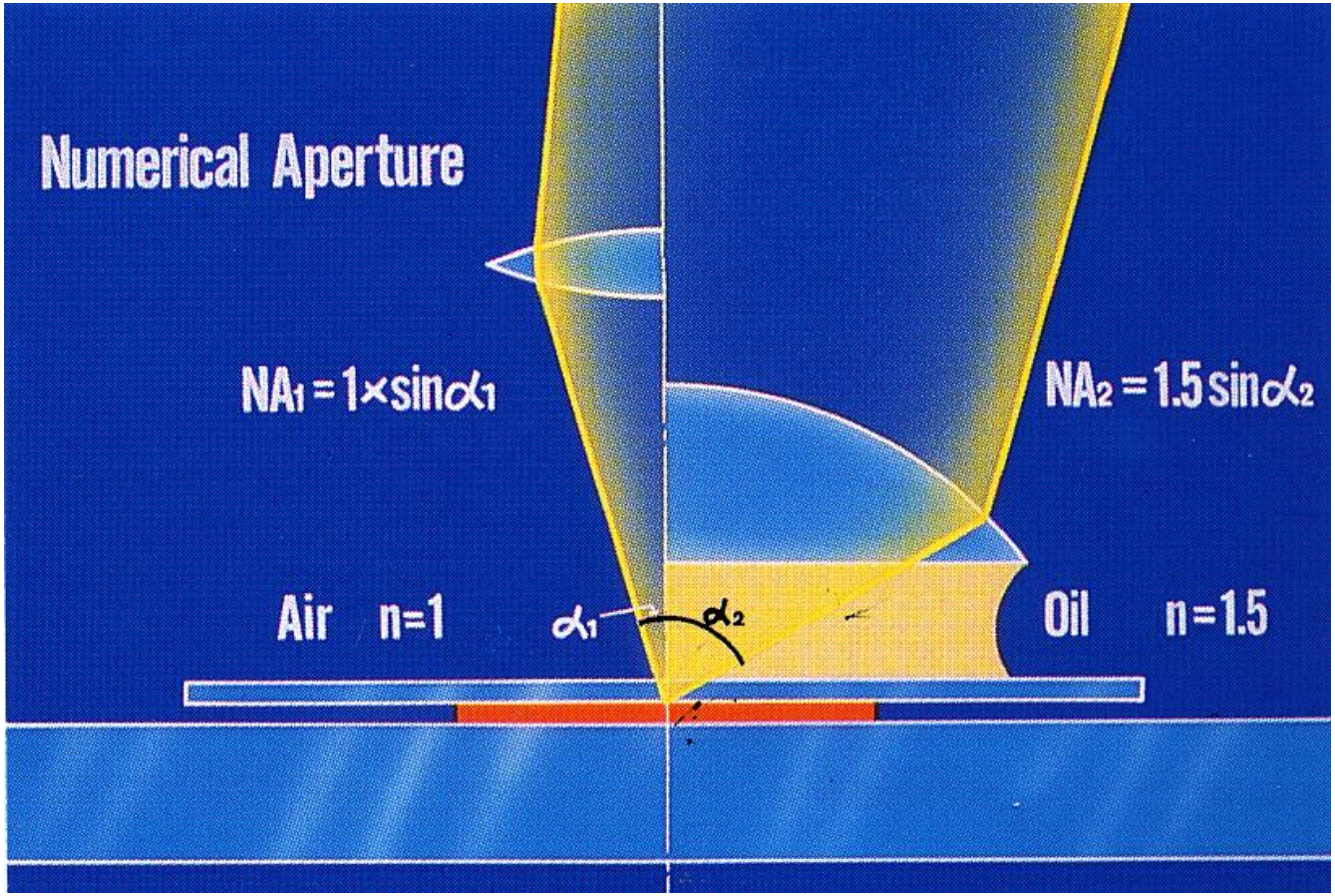


- Field of view: 20 mm
- Flatness: ★★★★★
- Color correction: ★★★★★

Apochromatically corrected immersion objectives for applications in physiology

介質的種類

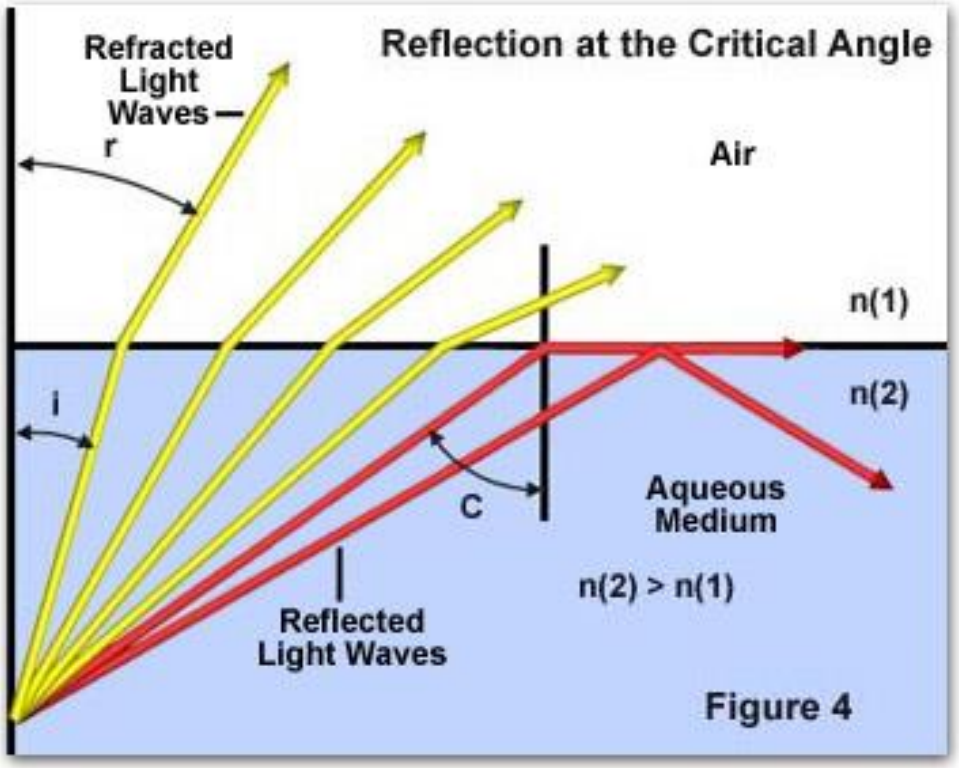
- 乾式物鏡
- 油鏡
- 水鏡



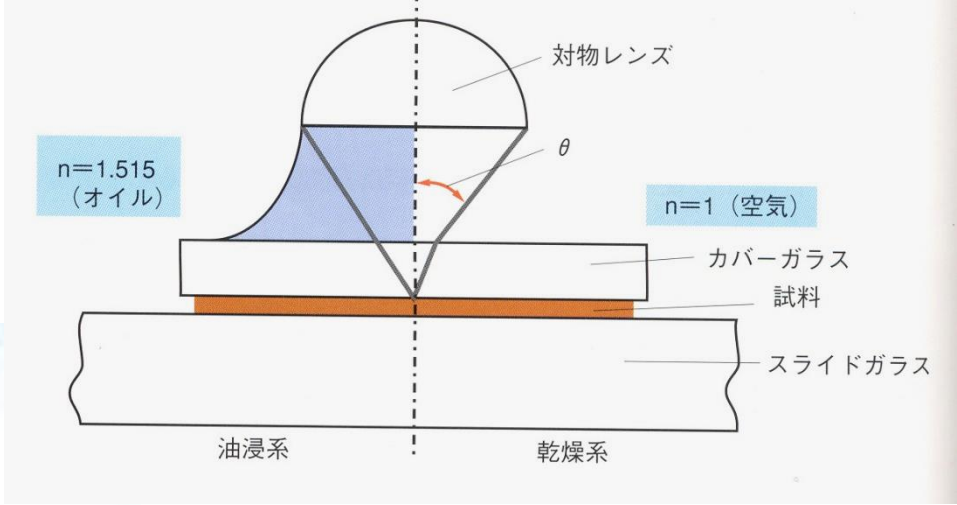
- **Measurements & artifacts: Message**

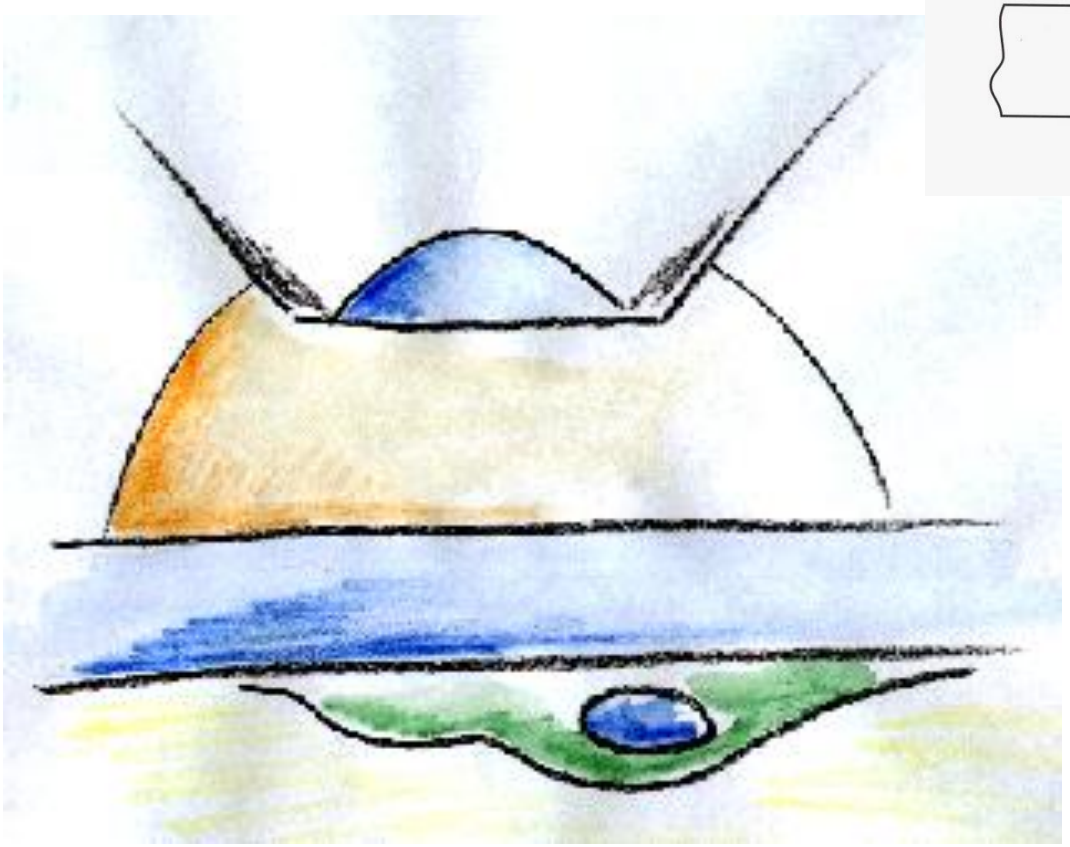
  - Refractive index mismatch drastically reduces resolution and leads to significant loss of intensity in fluorescence

  - 15µm cover slip deviation approximately kills half of the resolution and intensity



- **Observation and optical setup**





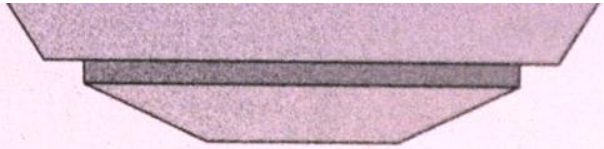
Micro objective

Immersion

Cover slip

Sample

The last 3 optical elements are usually added by the user !



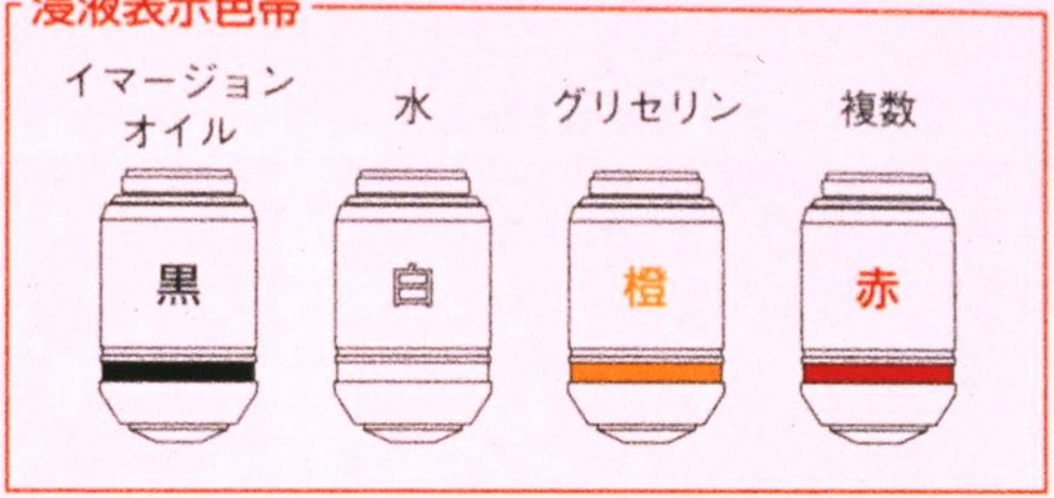
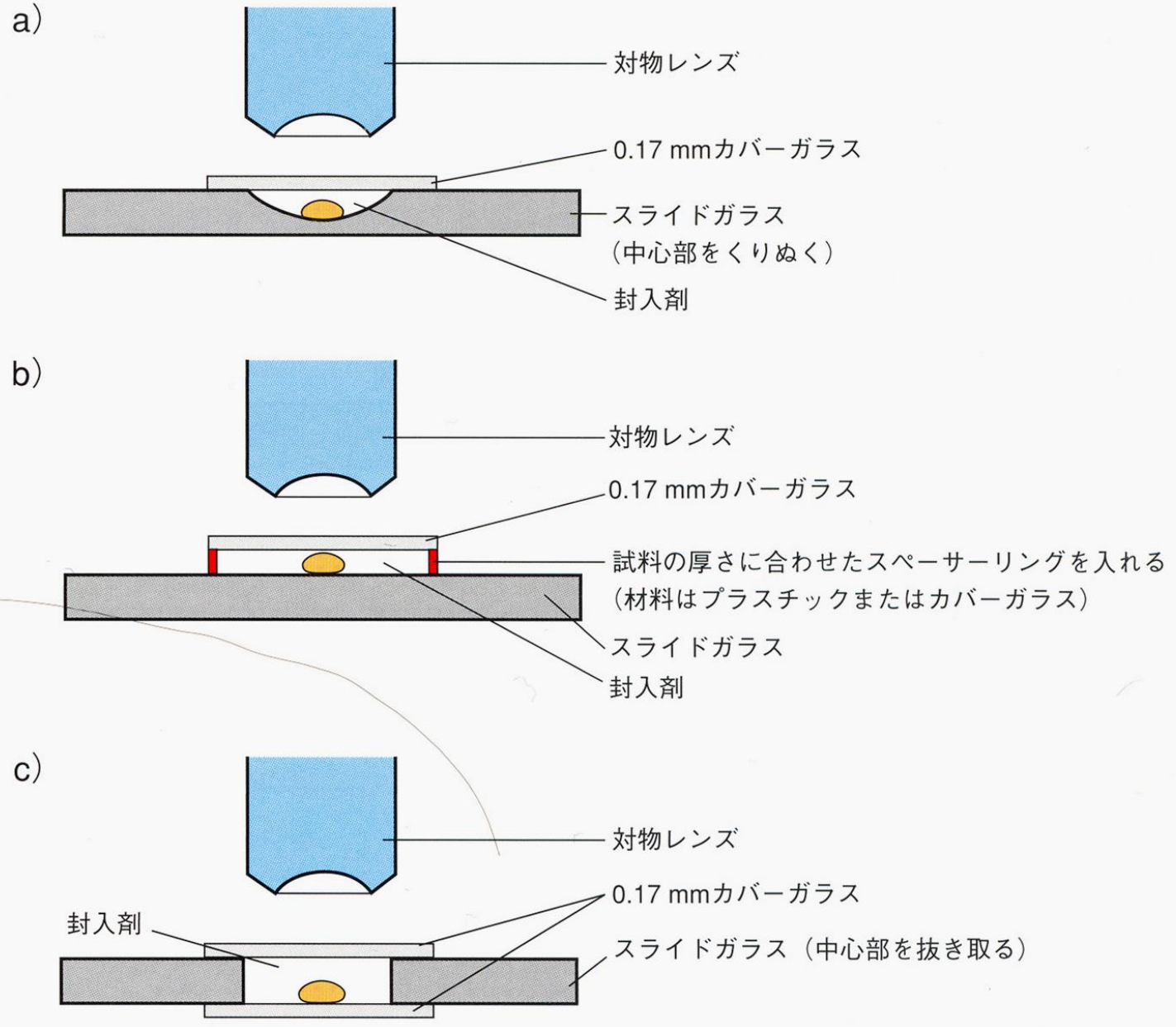


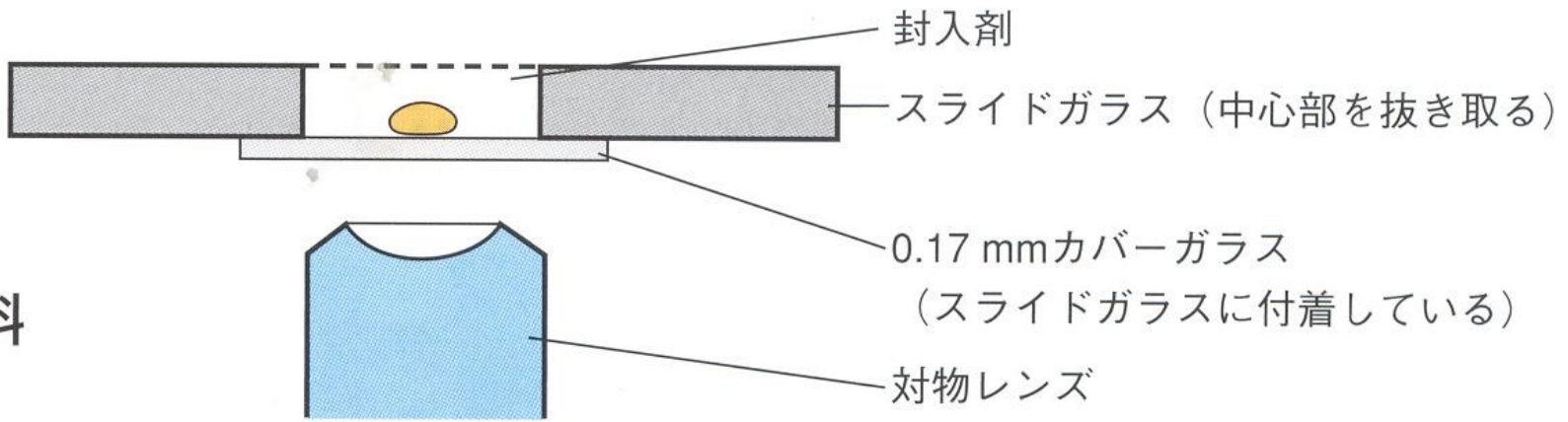
図3　対物レンズの外と内

A：60×のプランアポクロマート油浸対物レンズの外面．外面に記されている文字は，明視野観察用では

1）正立型顕微鏡の場合

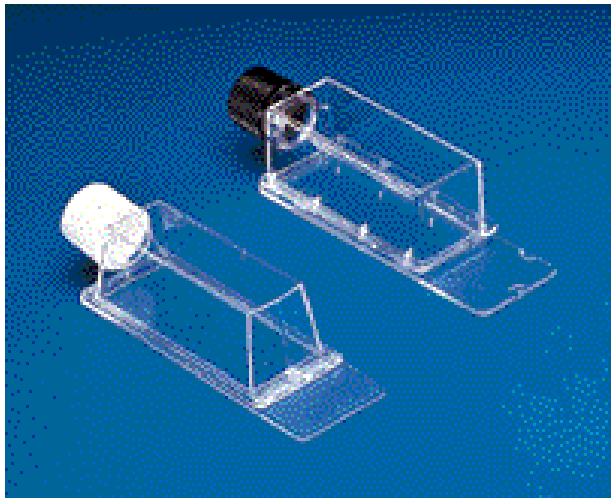

a)
対物レンズ
0.17 mmカバーガラス
スライドガラス
（中心部をくりぬく）
封入剤
b)
対物レンズ
0.17 mmカバーガラス
試料の厚さに合わせたスペーサーリングを入れる
（材料はプラスチックまたはカバーガラス）
スライドガラス
封入剤
c)
対物レンズ
0.17 mmカバーガラス
封入剤
スライドガラス（中心部を抜き取る）

2）倒立型顕微鏡の場合

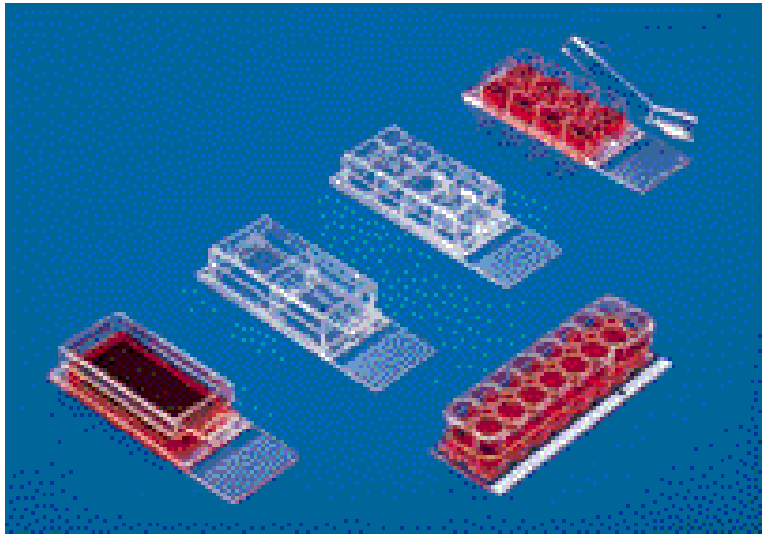


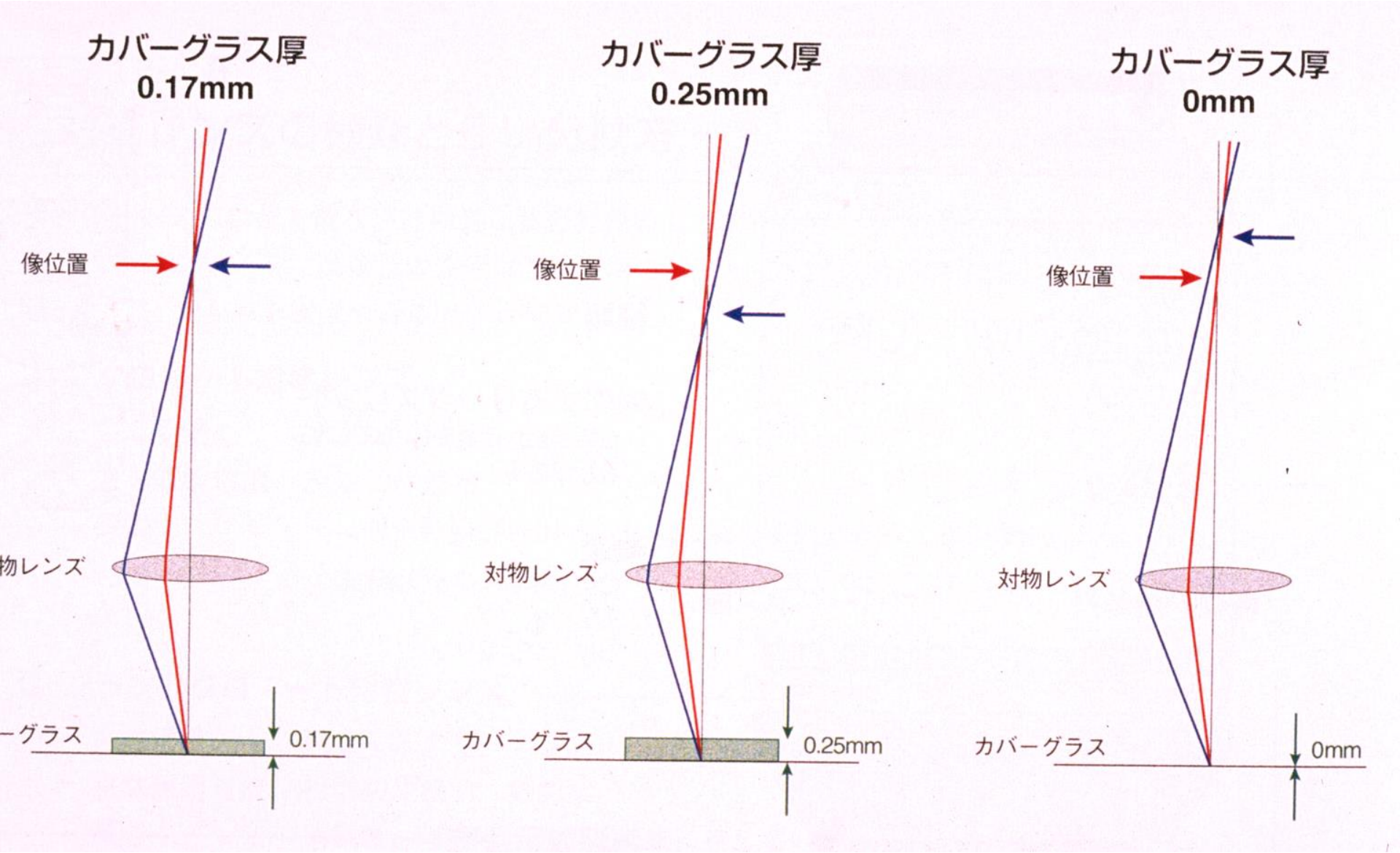
ための試料

NUNC FLASKETTE®
CHAMBER SLIDE/FLASKS

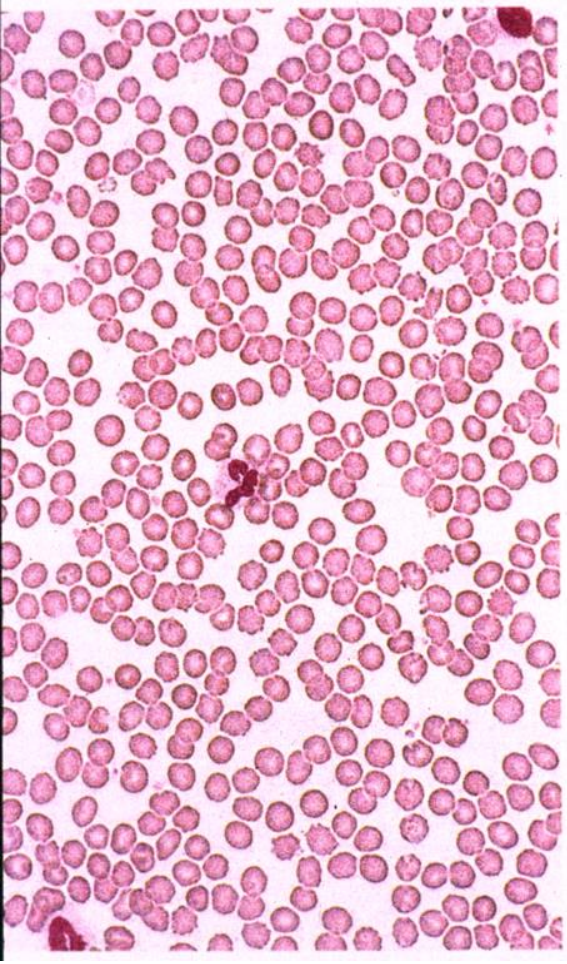


LAB-TEK® II
CHAMBERED COVERGLASS

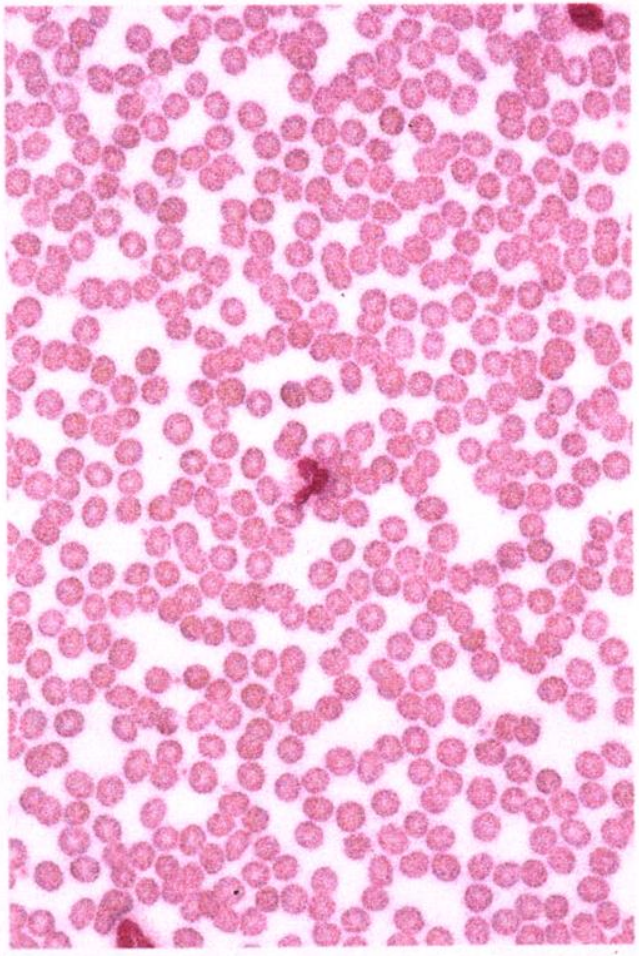



カバーグラス厚
0.17mm
像位置
物レンズ
ーグラス
0.17mm
カバーグラス厚
0.25mm
像位置
対物レンズ
カバーグラス
0.25mm
カバーグラス厚
0mm
像位置
対物レンズ
カバーグラス
0mm

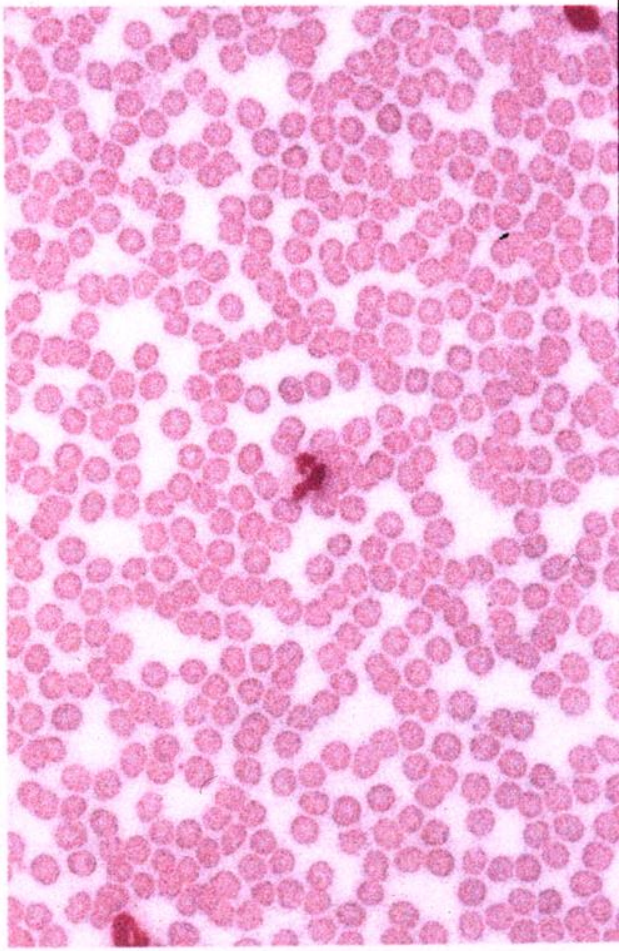
カバーグラス厚 0.17mm



カバーグラス厚 0.25mm

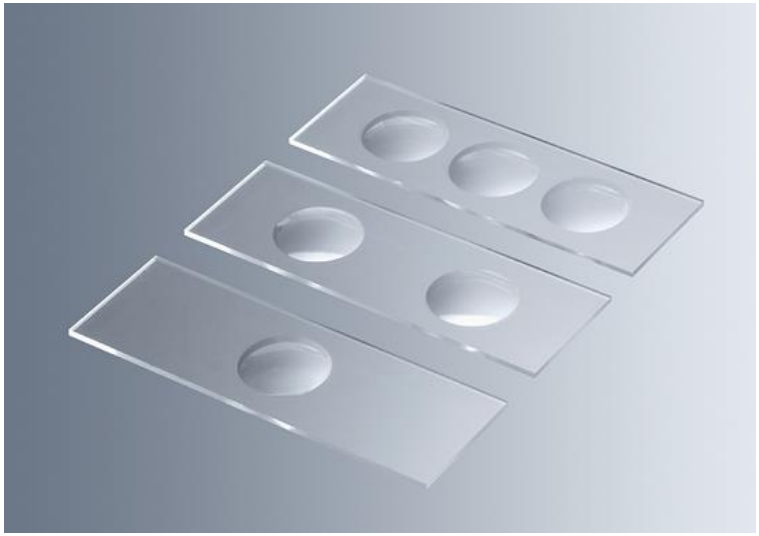


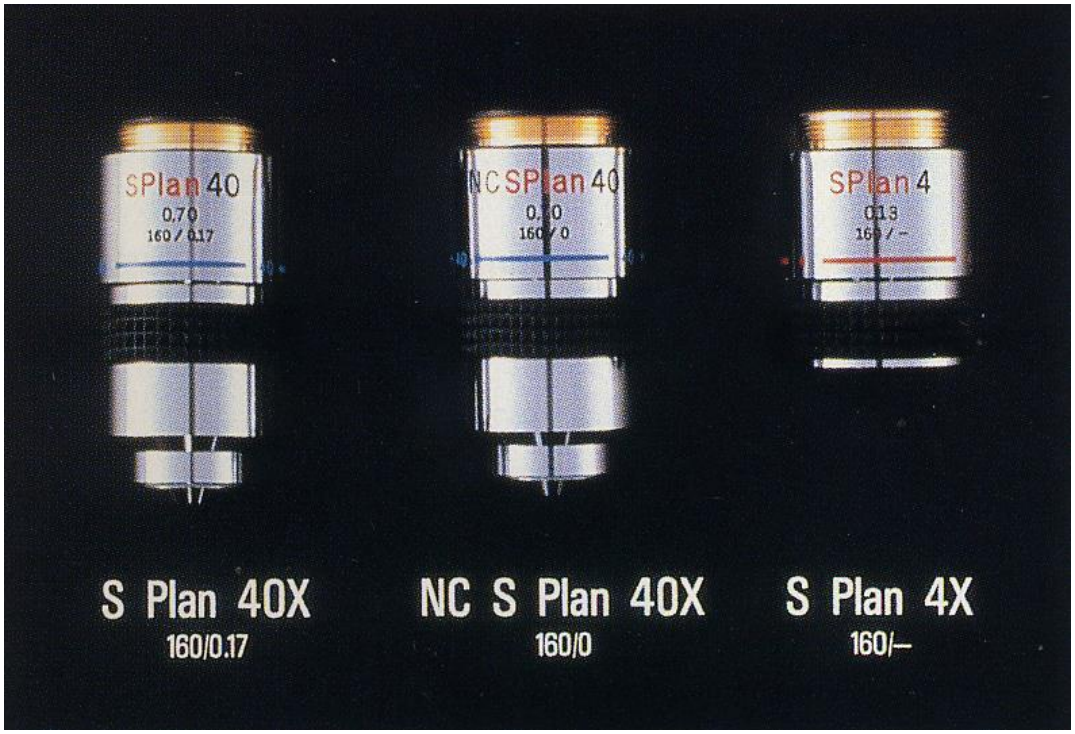
カバーグラス厚 0mm



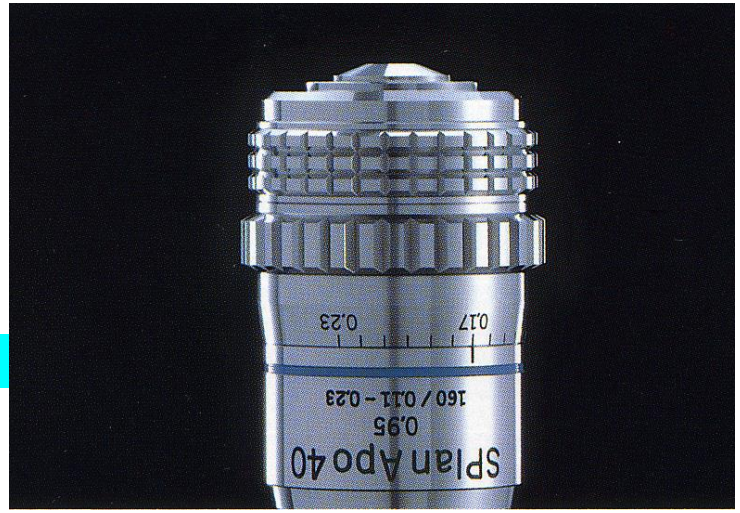
修正蓋玻片的物鏡

- 開口值物鏡需要可調整蓋玻片厚度，以取得最佳影像．(0.11-0.23mm)　　Depression slide



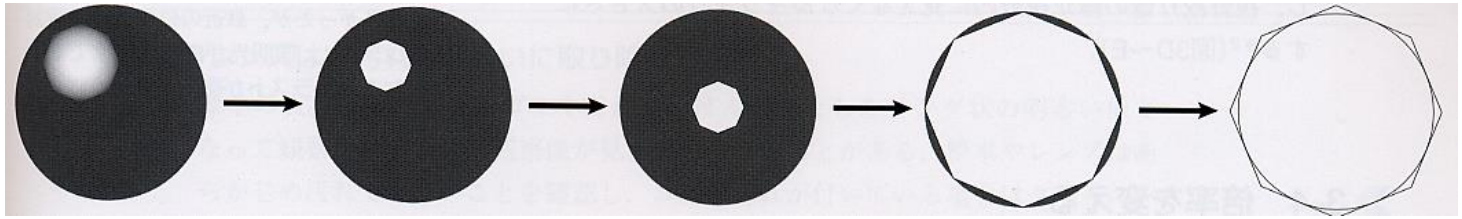


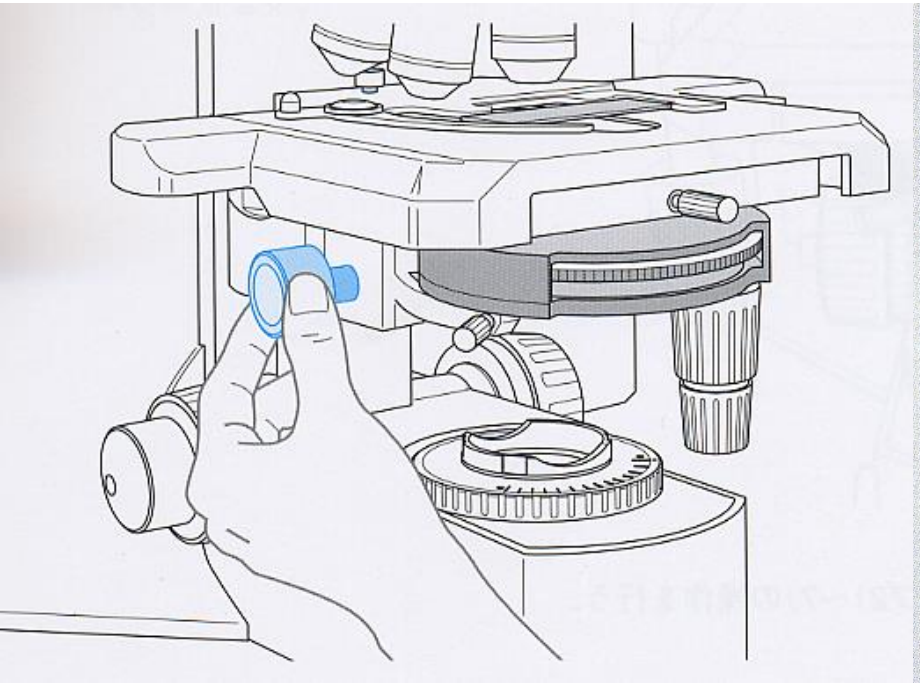
使用0.17mm 蓋玻片　不使用蓋玻片　蓋玻片厚度無關

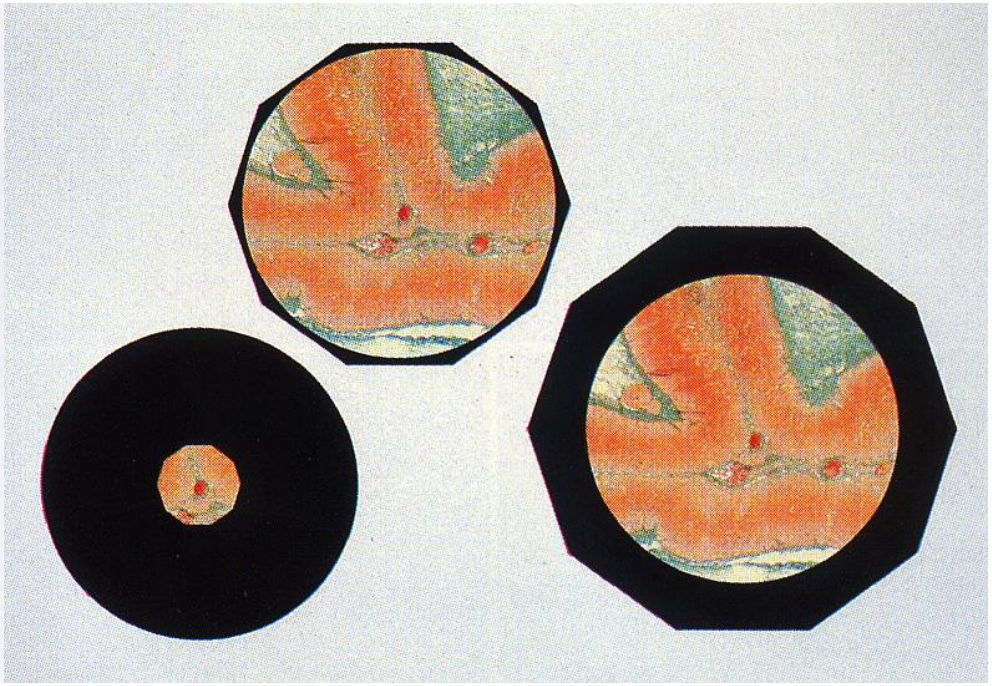


凱勒照明調整目的:達到最均勻的照明

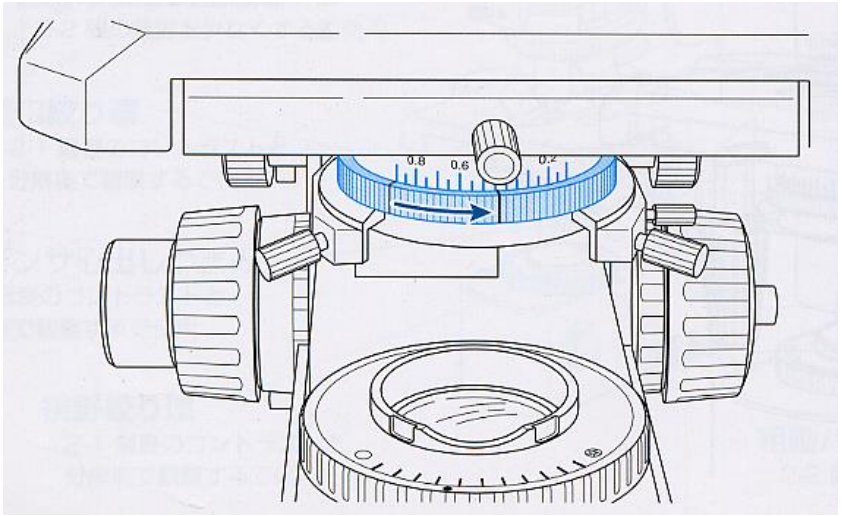
- 將標本焦距與視野光圈調至同時清晰可見

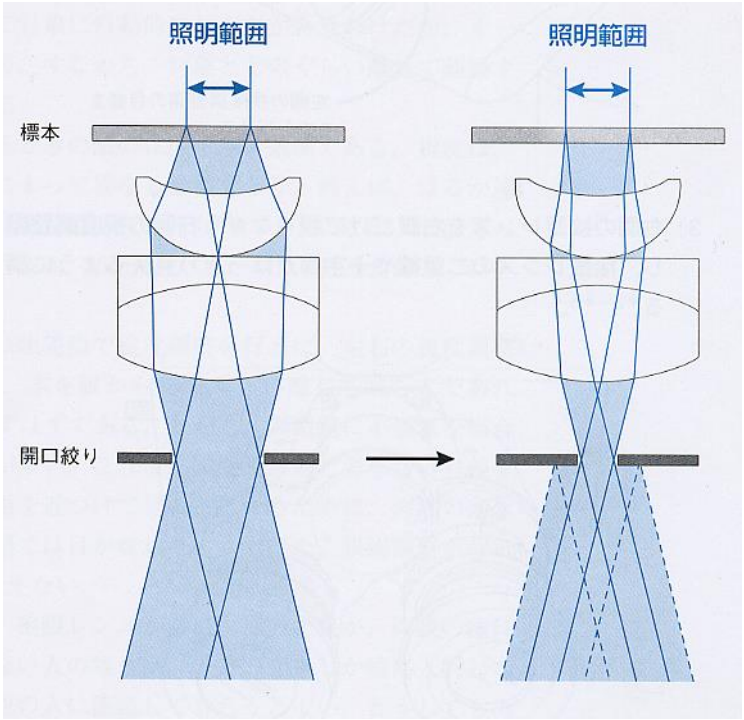


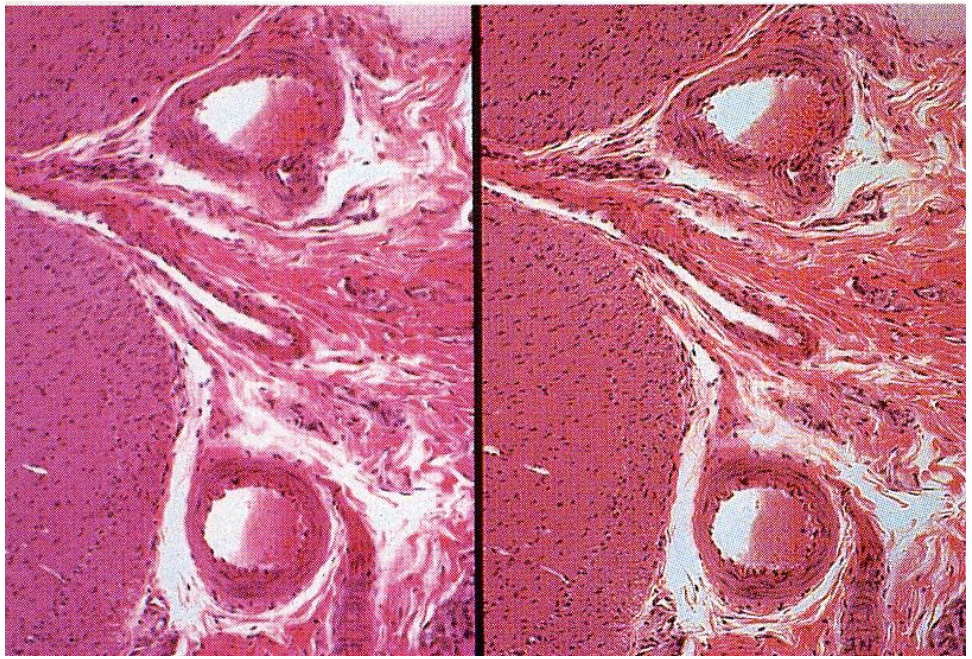




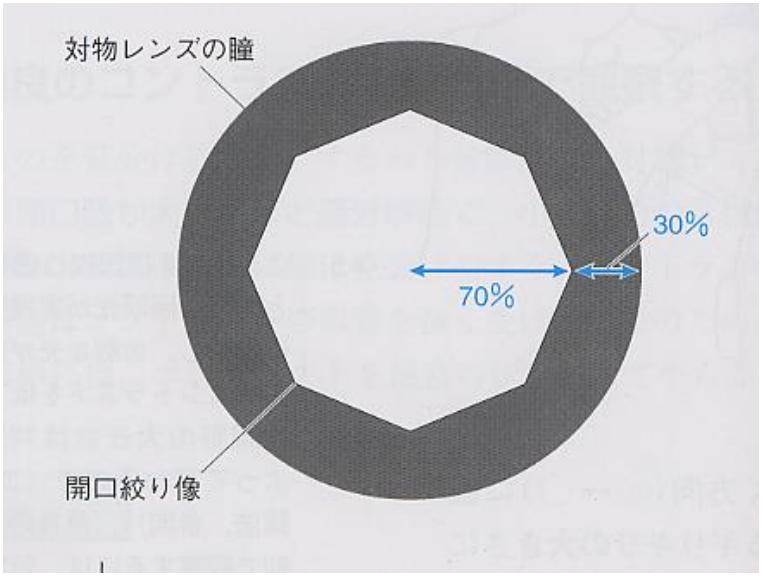
聚光鏡：決定影像的解晰度與對比的工具

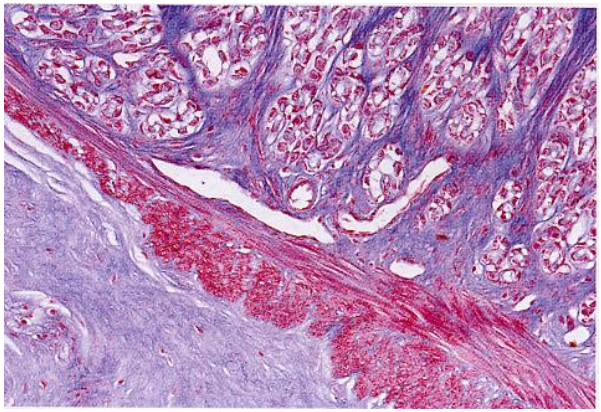


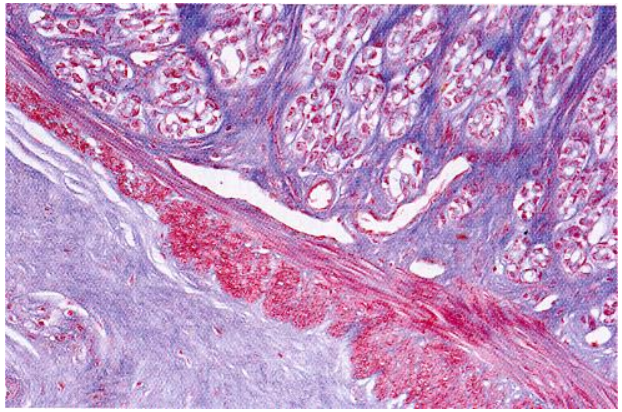


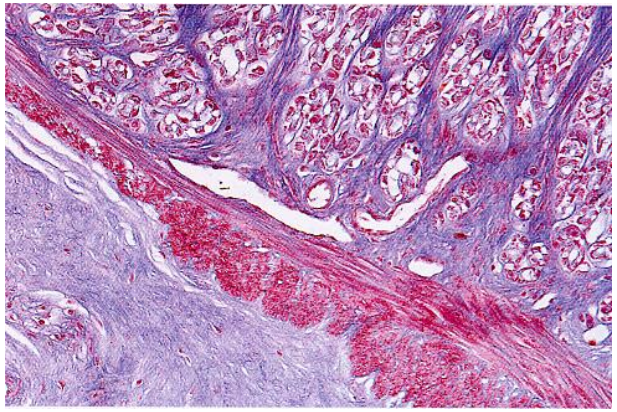


聚光鏡的調整: 一般而言聚光鏡的開口值約為物鏡開口值的70%-80%

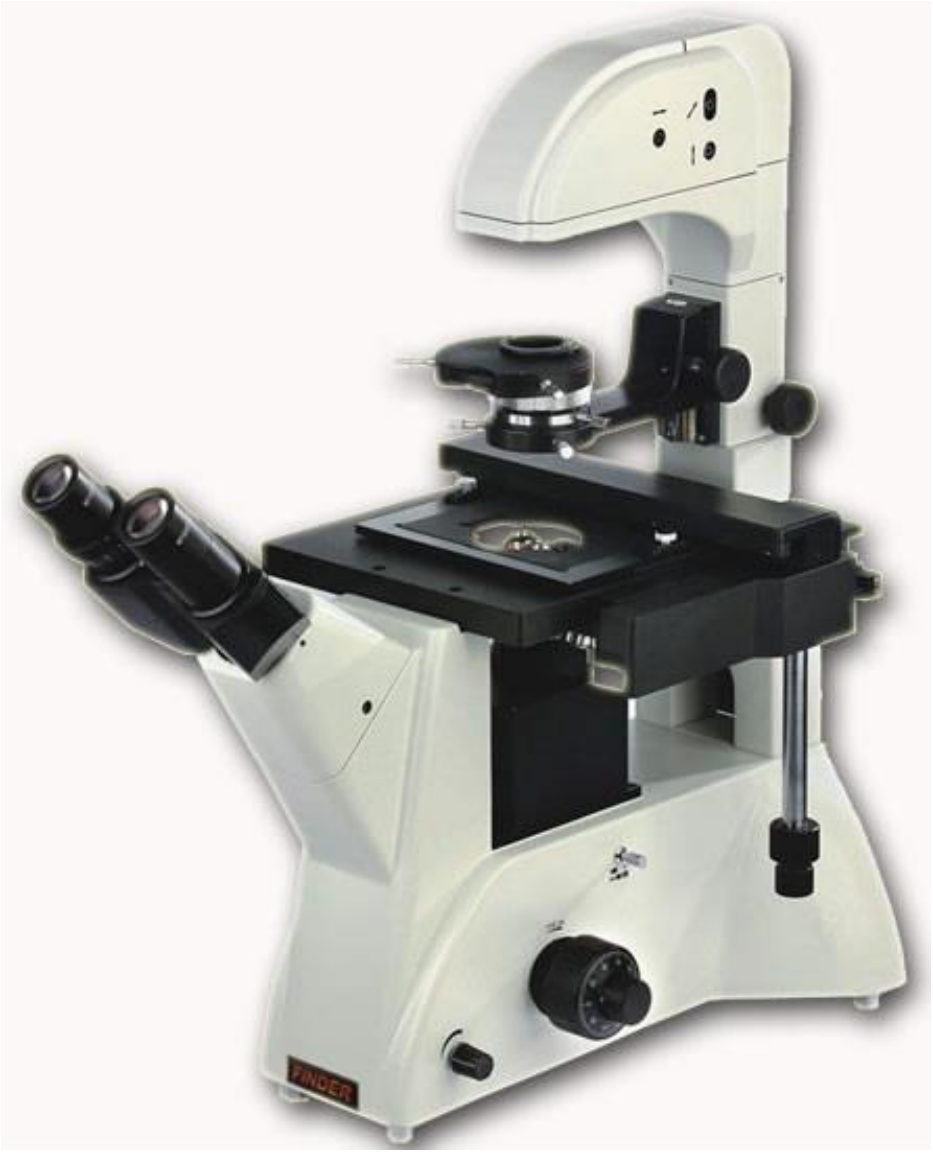


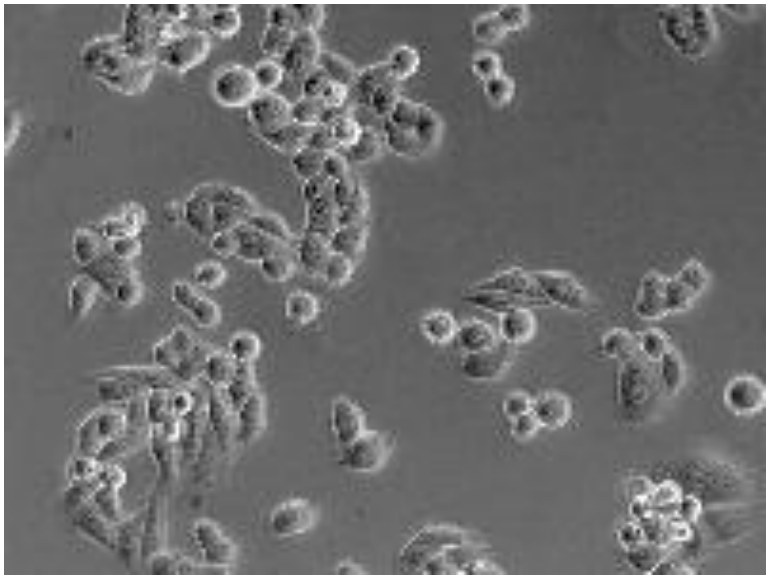


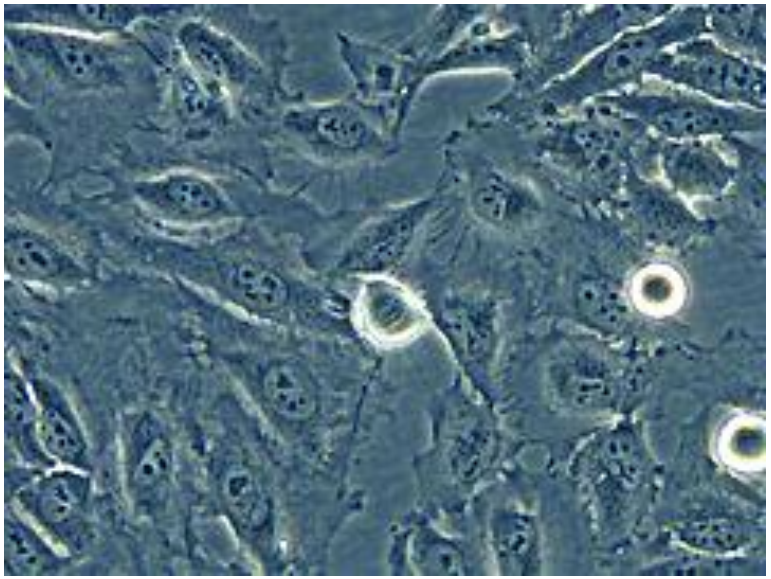




位相差顯微鏡 (Phase Contrast)





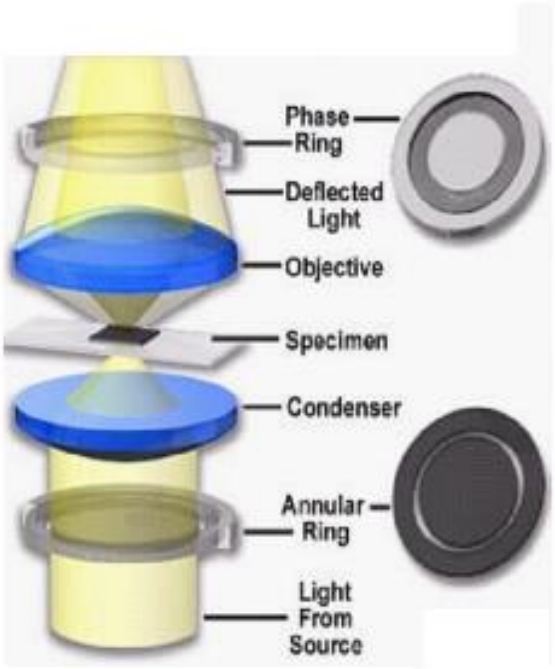


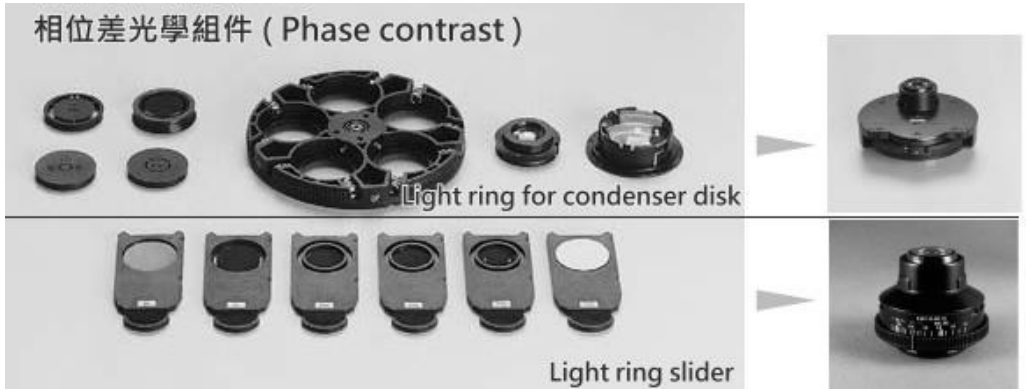
位相差的配件

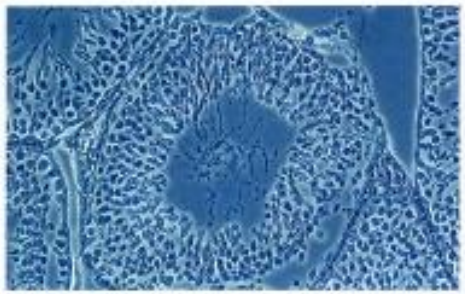
- 位相差專用物鏡
- 位相差環
- 萬用聚光鏡
- 調整用目鏡

Light rings PH0, PH1, PH2

Phase contrast 光路 / 光學組件



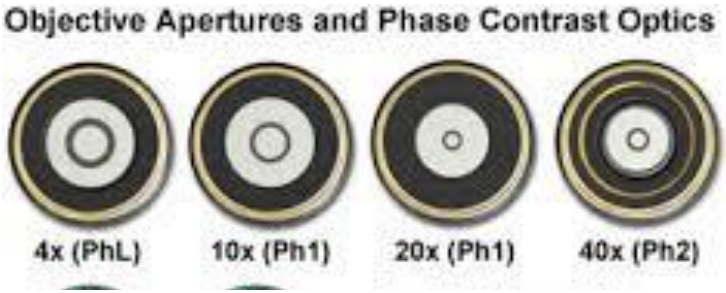


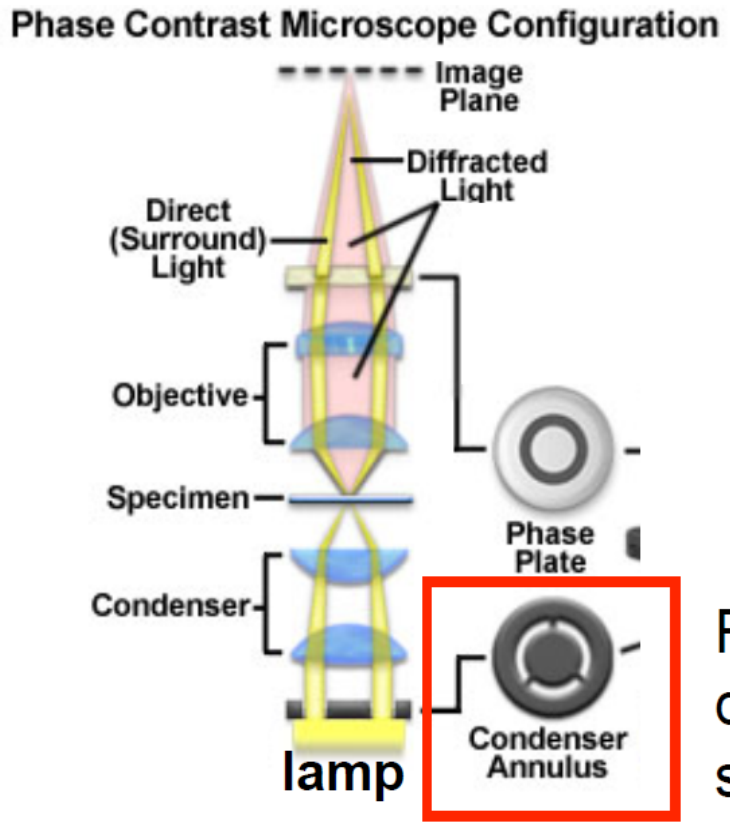


Phase contrast

DIC

位相差的原理

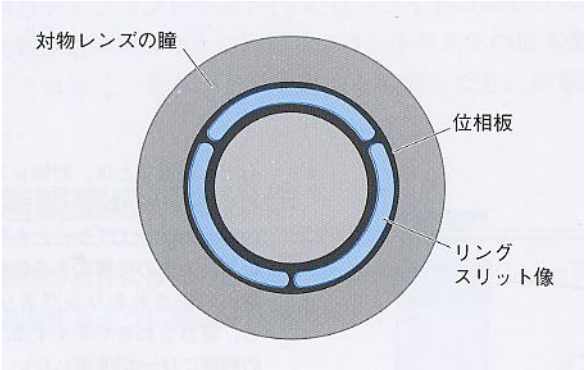




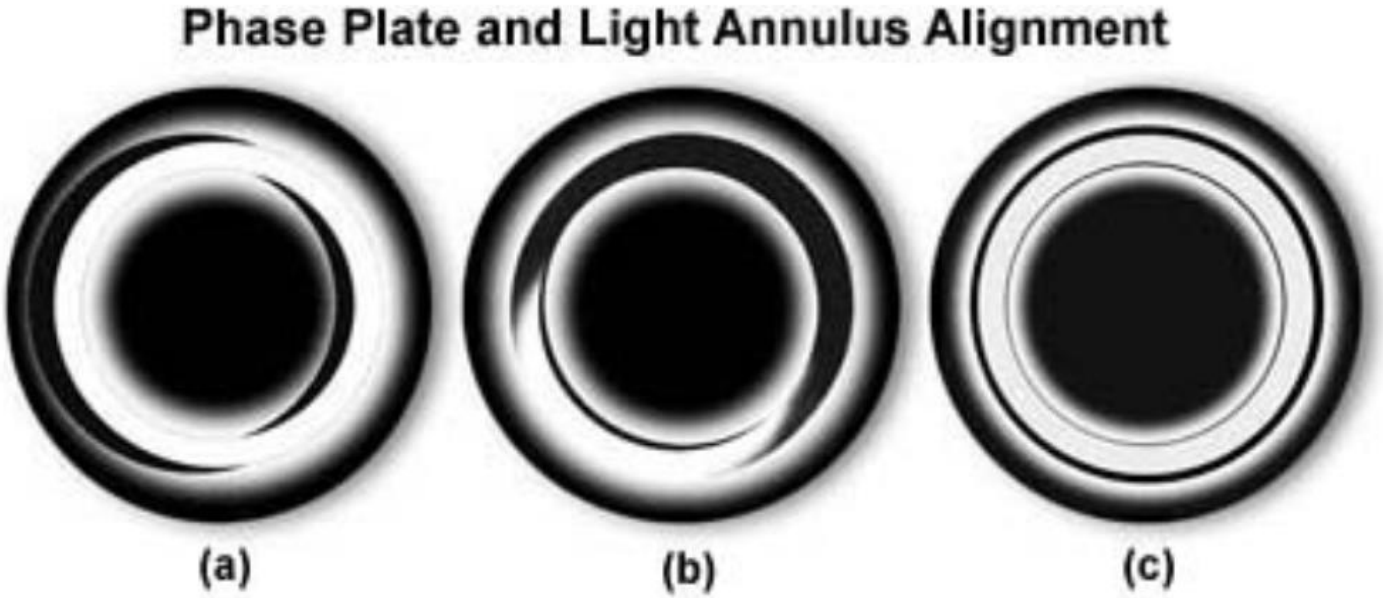
Restricts angles of illumination so diffracted and undiffracted light can be selectively modulated at phase plate

位相差的調整

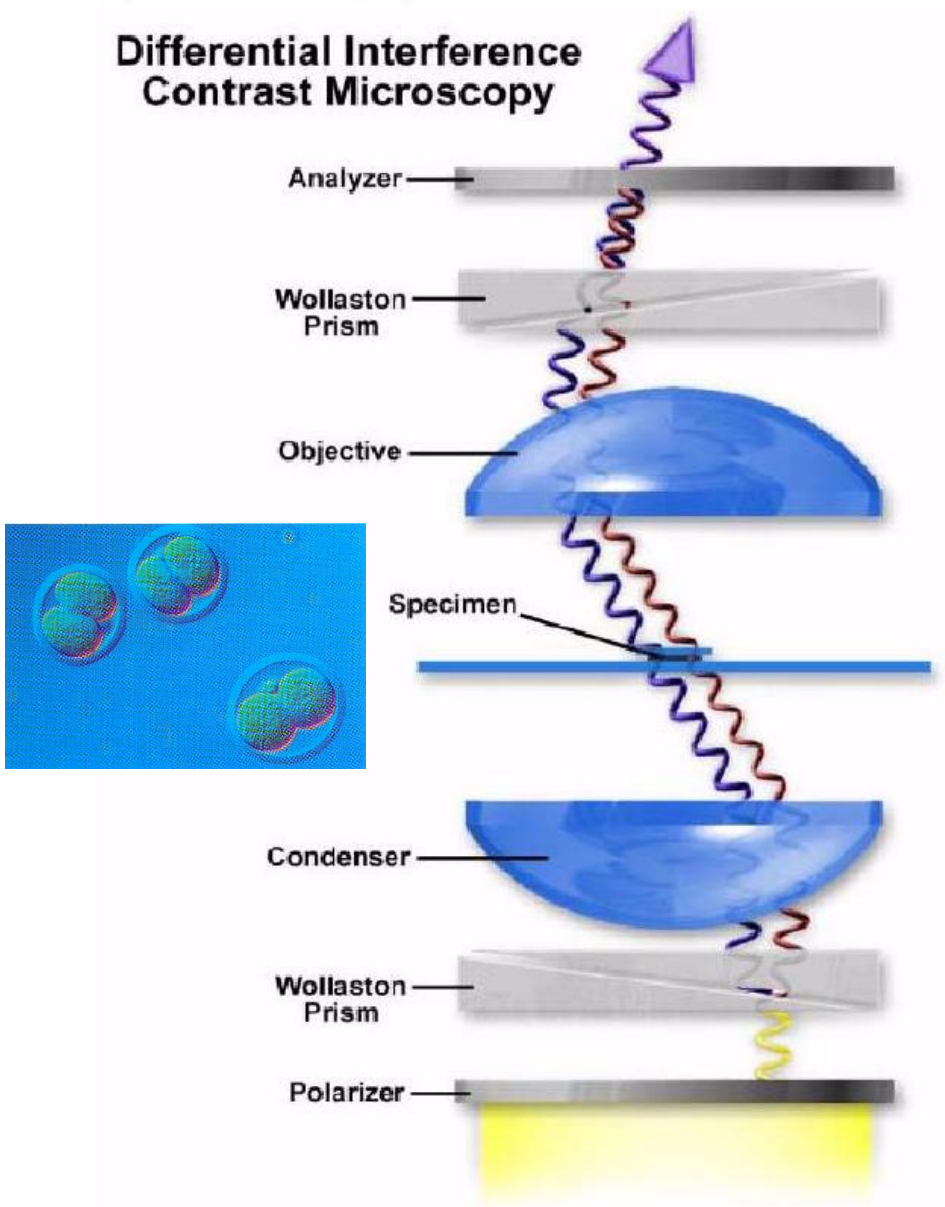
- 將每一倍率物鏡的亮環及暗環調整為重疊



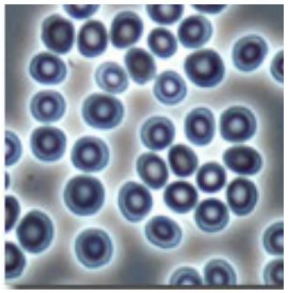
PHASE CONTRAST ALLIGNMENT



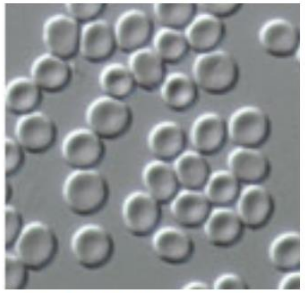
微分干涉顯微鏡



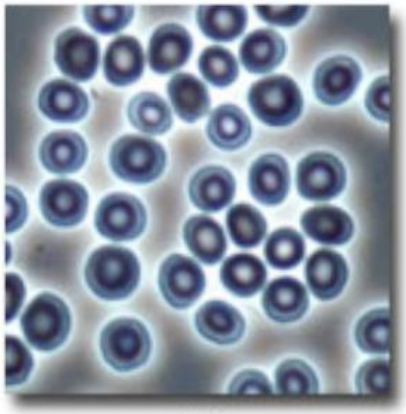
DIC: an alternative technique for enhancing contrast



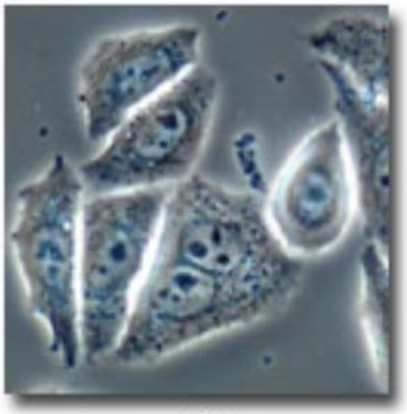
Phase



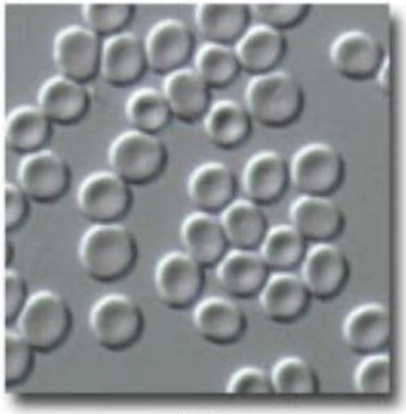
DIC (Differential Interference Contrast)



(a)



(c)



(b)

(d)

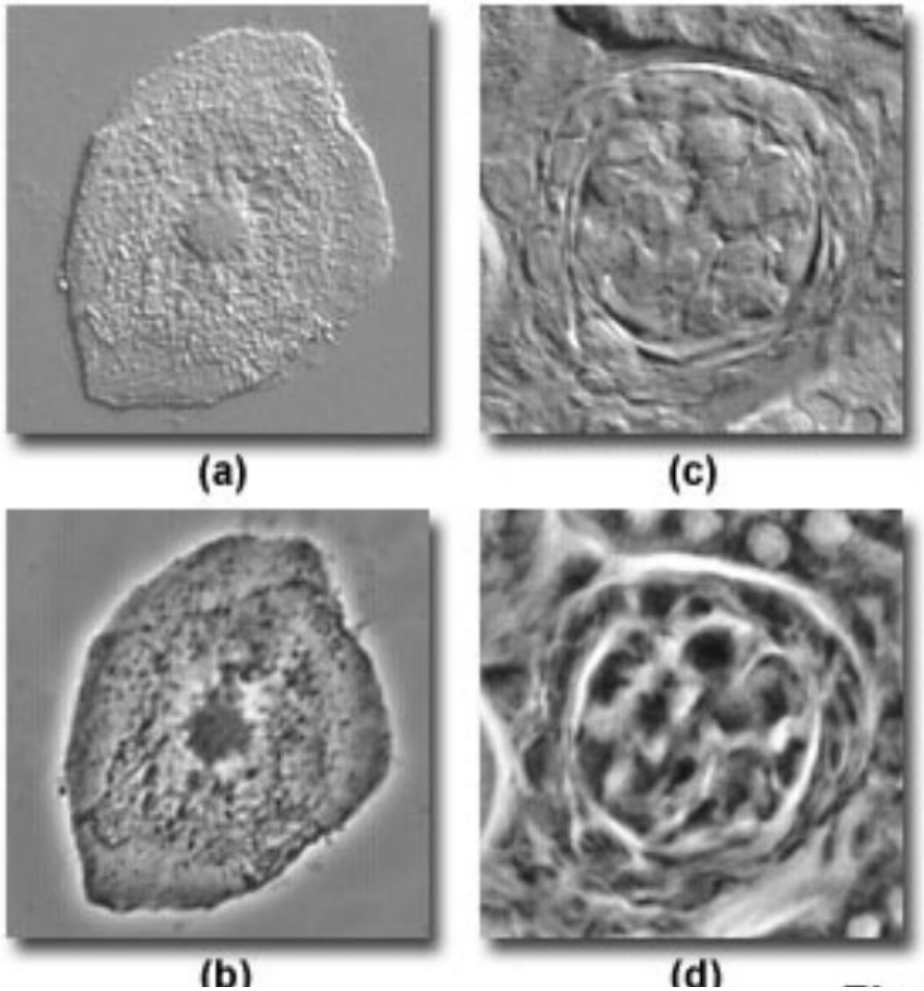
微分干涉顯微鏡的配件

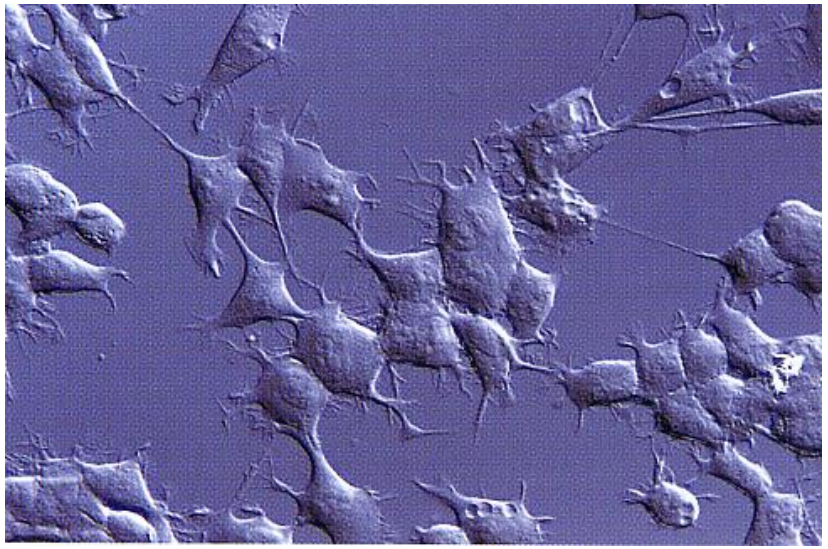
- 萬物聚光鏡
- 微分干涉鍥鏡
- 起偏鏡
- 檢偏鏡

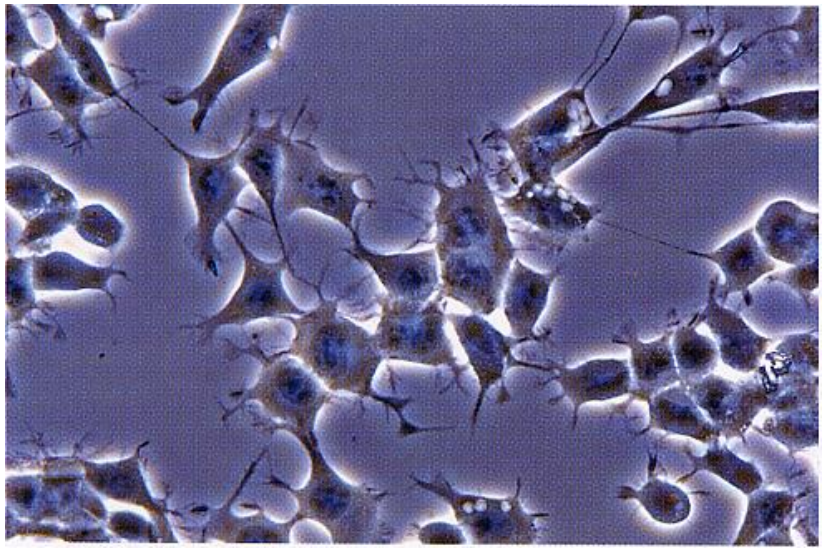
微分干涉 位相差 的比較

	位相差	微分干涉
對比方式	以標本厚度轉換成對比	以厚度變化轉換成對比
對比的調整	無法調整	以錂鏡調整
影像的特性	以明暗度表現	以立體感及明暗表現
解析度	比微分干涉差	高
合適的標本	細微構造 標本厚度10μm	細微構造，外觀． 標本厚度數百μm
容器	可使用塑膠類	不可使用塑膠類

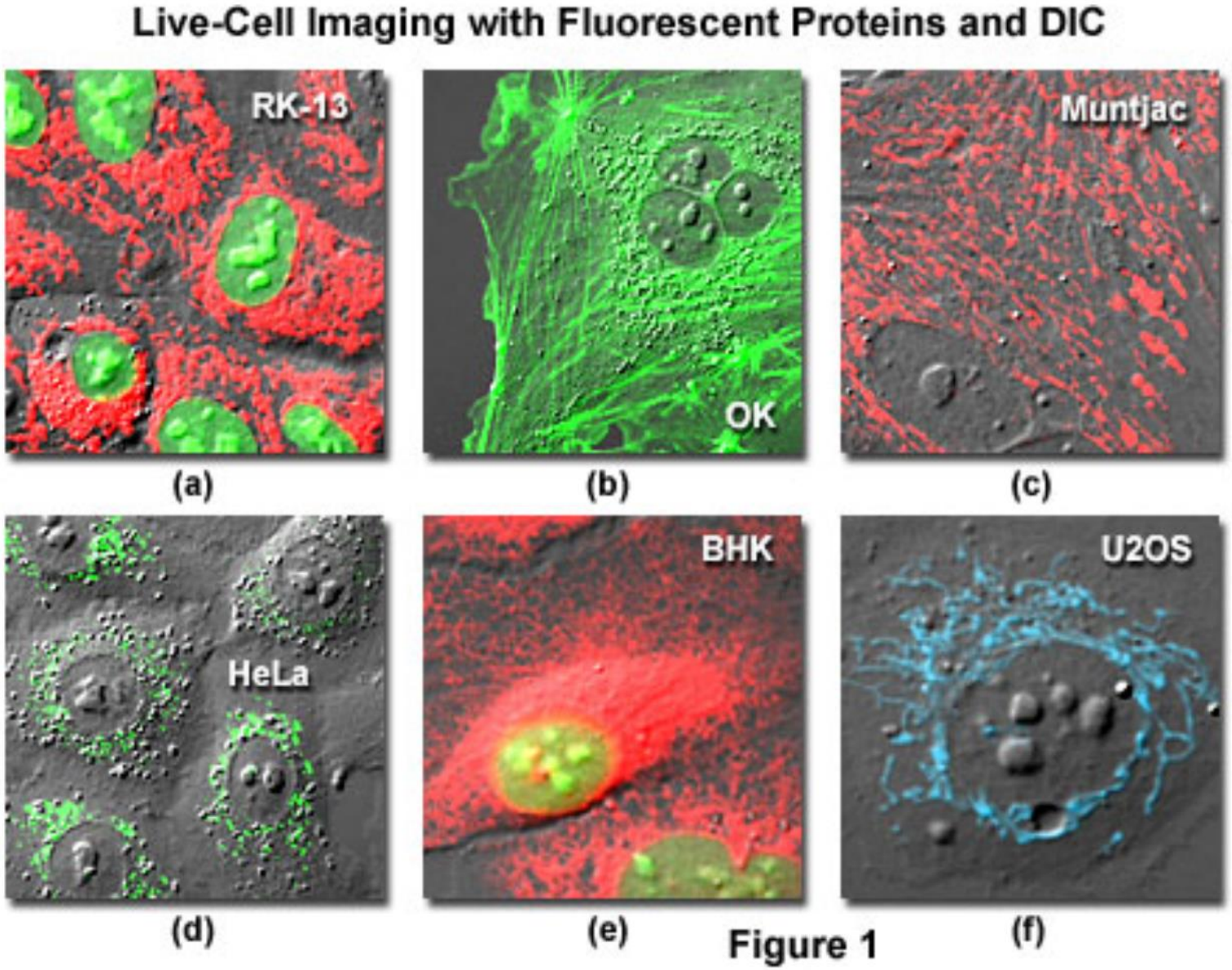
微分干涉及位相差的實例







微分干涉及位相差的實例



螢光顯微鏡
(Fluorescence microscope)

A **fluorescence microscope** is an optical microscope that uses fluorescence instead of, or in addition to, scattering, reflection, and attenuation or absorption, to study the properties of organic or inorganic substances.

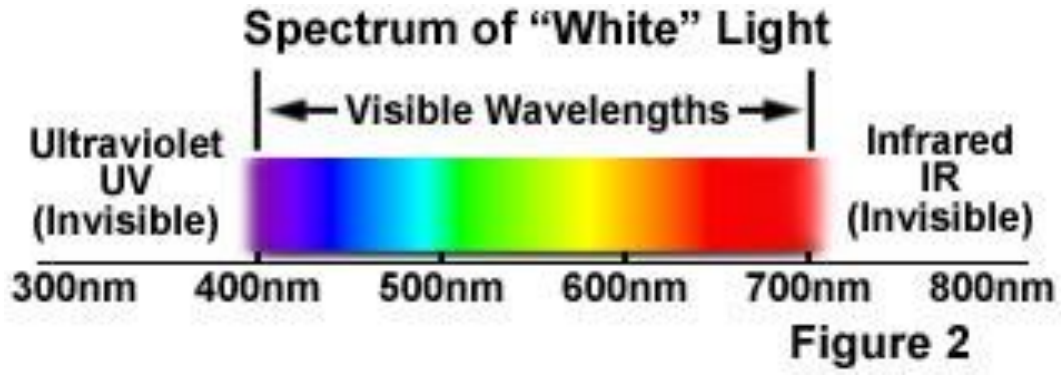


Figure 2

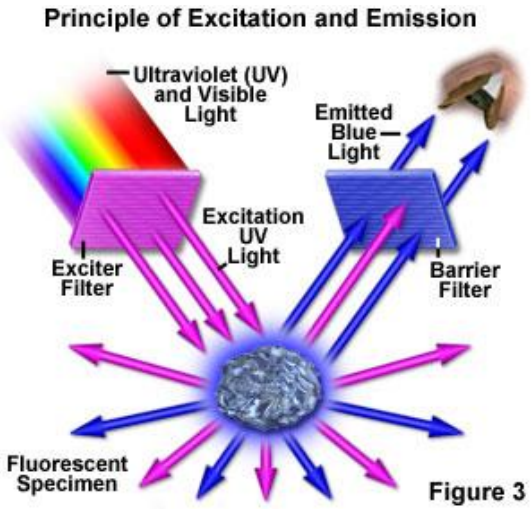
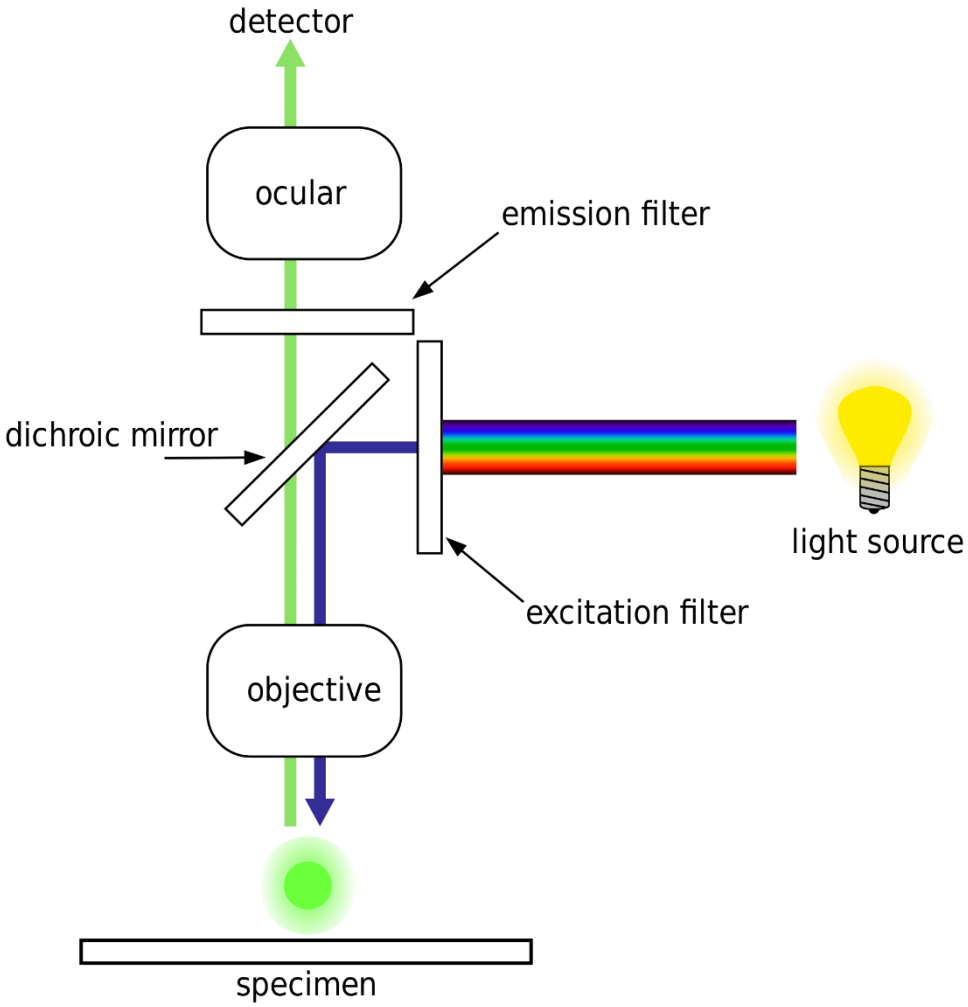


Figure 3

Fluorescence microscope



Schematic of a fluorescence microscope.

An upright fluorescence microscope (Olympus BX61) with the fluorescence filter cube turret above the objective lenses, coupled with a digital camera.

Figure 1 - Epi-Fluorescence Microscope

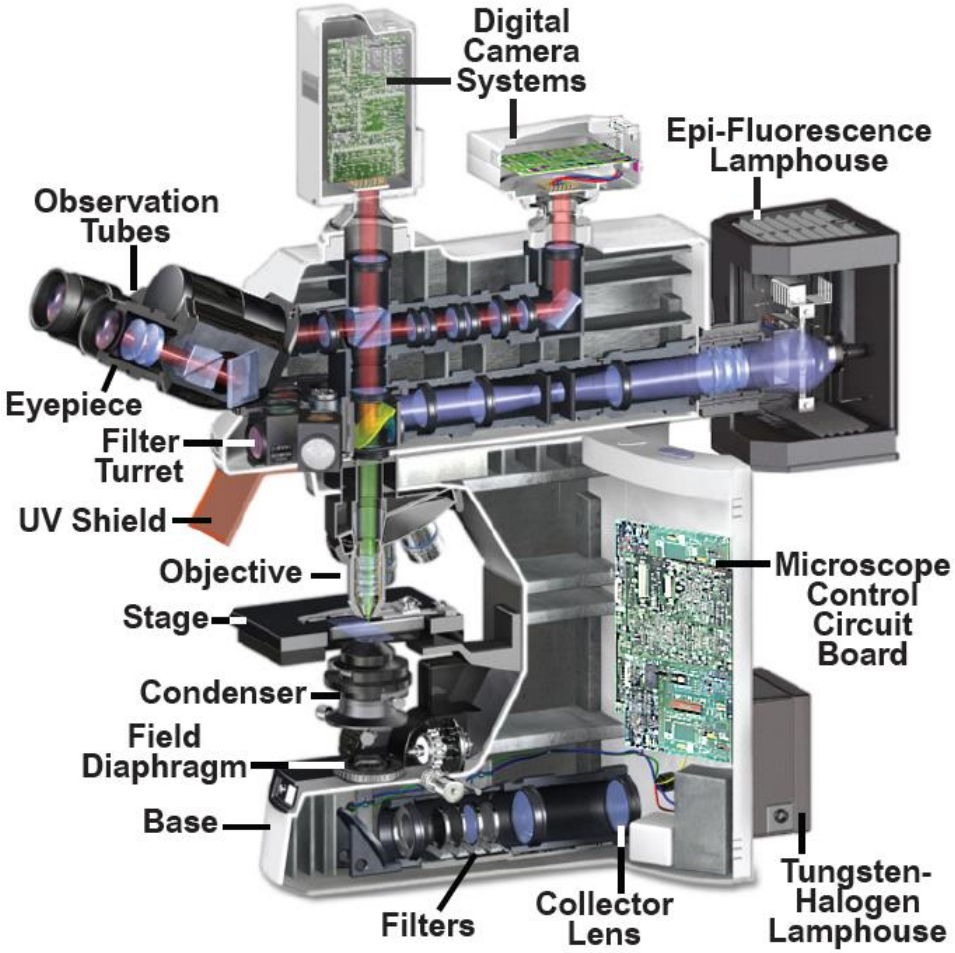


Figure 2 - Fluorescence Filters

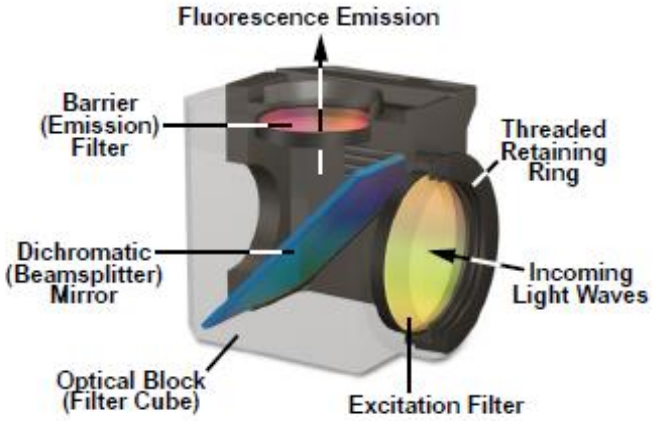
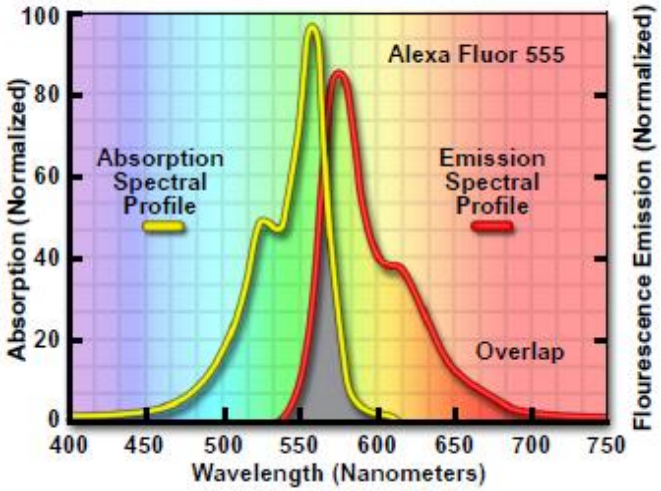


Figure 3 - Fluorophore Absorption and Emission Profiles



Light sources

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide.[4] Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs.

Mercury Arc Lamp

Figure 1 *- Arc Discharge Lamp Focus and Alignment Sequence*

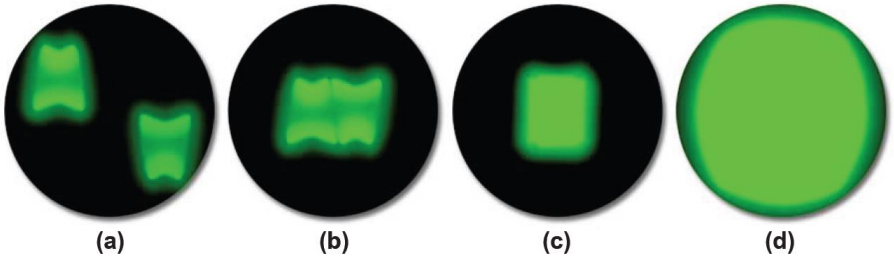
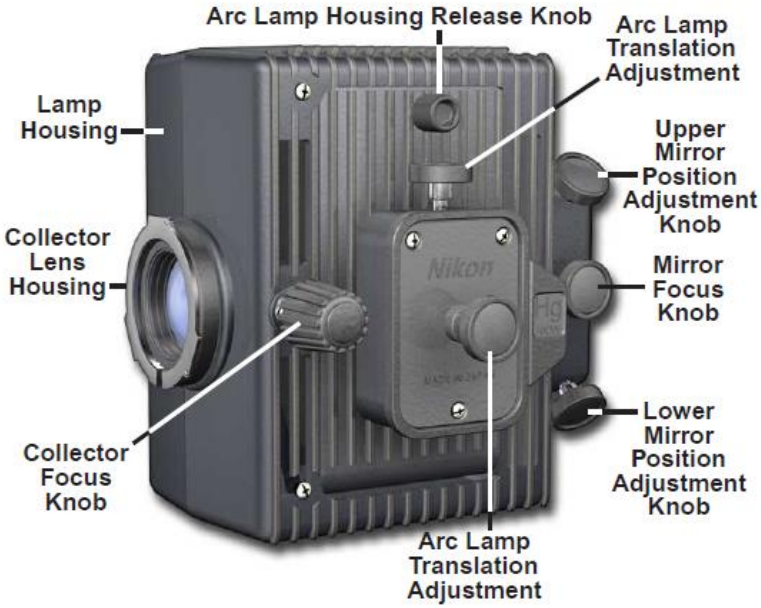


Figure 3 *- Nikon HMX-4 Mercury/Xenon Lamphouse*



Sample preparation

1. Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains such as DAPI and Hoechst (excited by UV wavelength light).
2. Immunofluorescence is a technique which uses the highly specific binding of an antibody to its antigen in order to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody specific for the molecule of interest. A fluorophore can be directly conjugated to the primary antibody. Alternatively a secondary antibody, conjugated to a fluorophore, which binds specifically to the first antibody can be used.

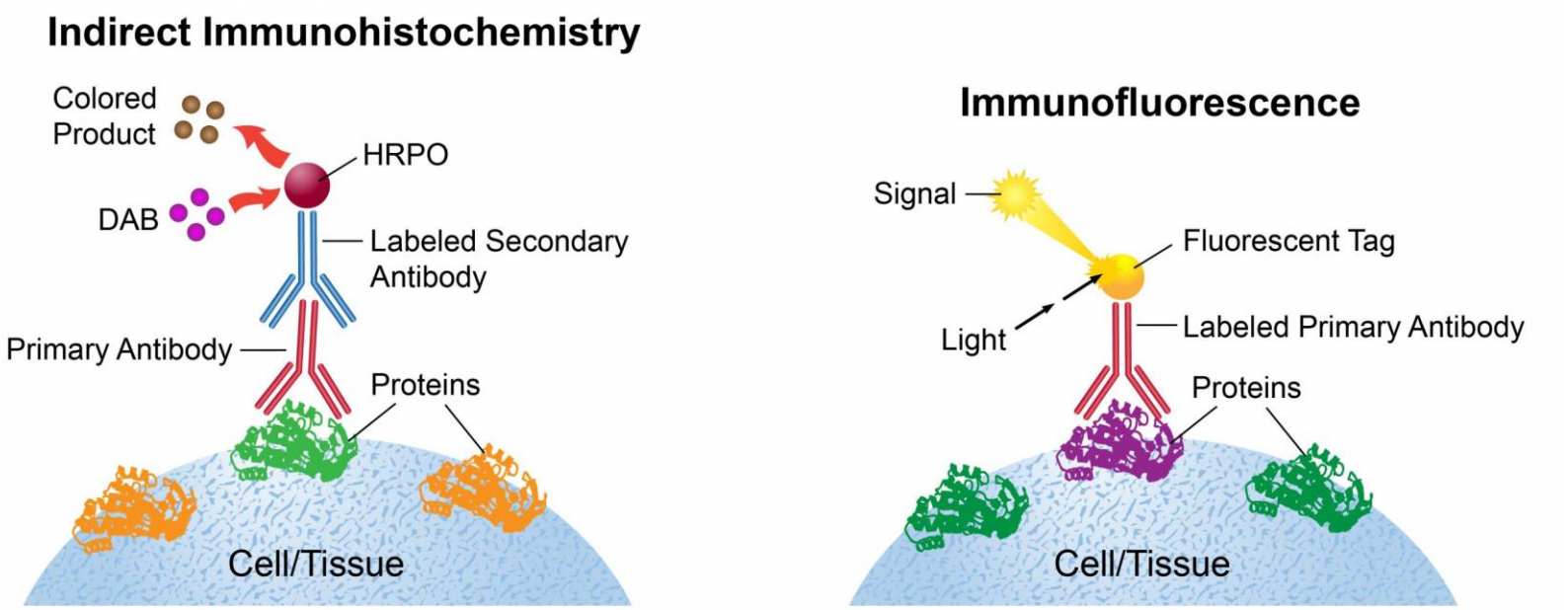
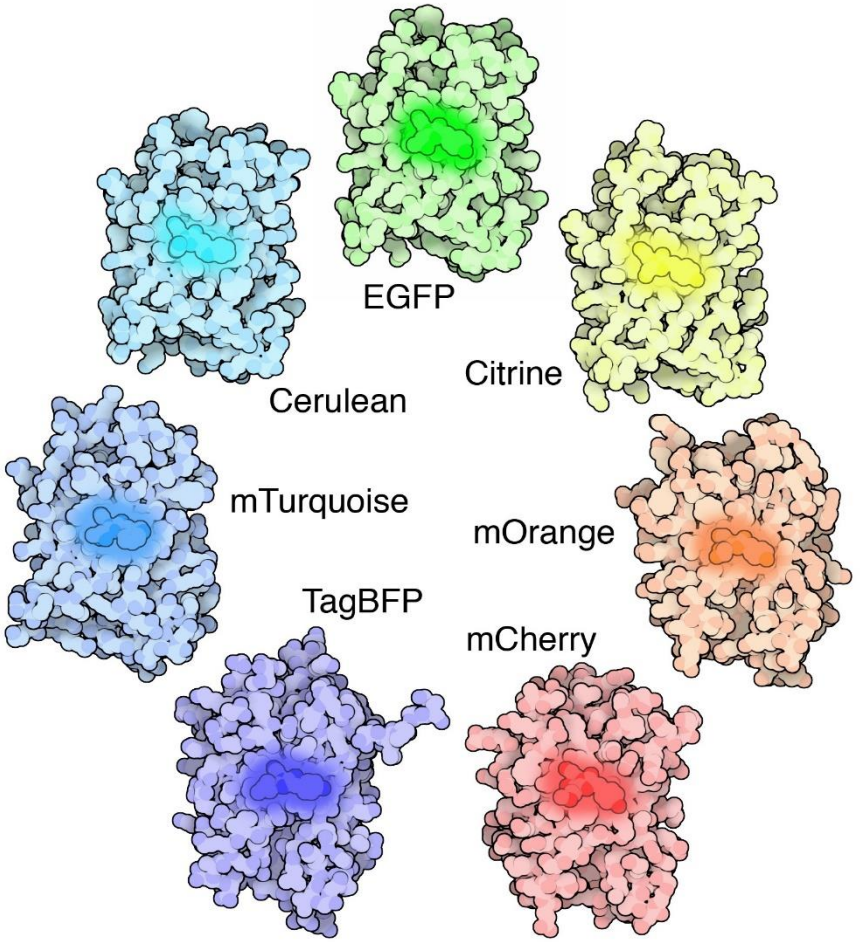


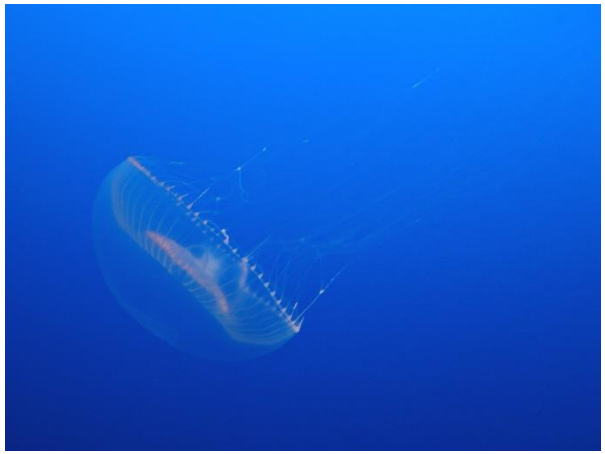
Diagram 1: *Illustration of Indirect Immunohistochemistry and Immunofluorescence methods.*

Green Fluorescent Protein (GFP)

is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. Similar proteins that also fluoresce green are found in many marine organisms, but the label *GFP* traditionally refers to this particular protein, which was first isolated from the jellyfish *Aequorea victoria* and is sometimes called—when such precision is required—*avGFP*.

Scientists Roger Y. Tsien, Osamu Shimomura, and Martin Chalfie were awarded the 2008 Nobel Prize in Chemistry on 10 October 2008 for their discovery and development of the green fluorescent protein.





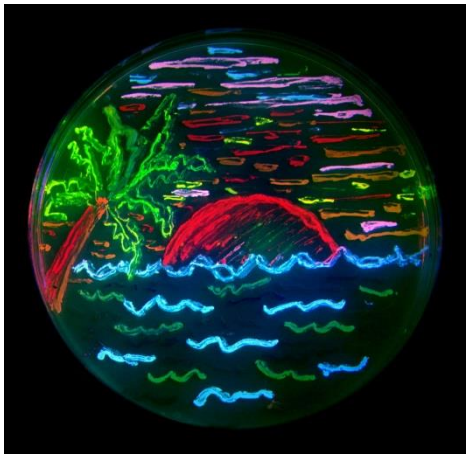
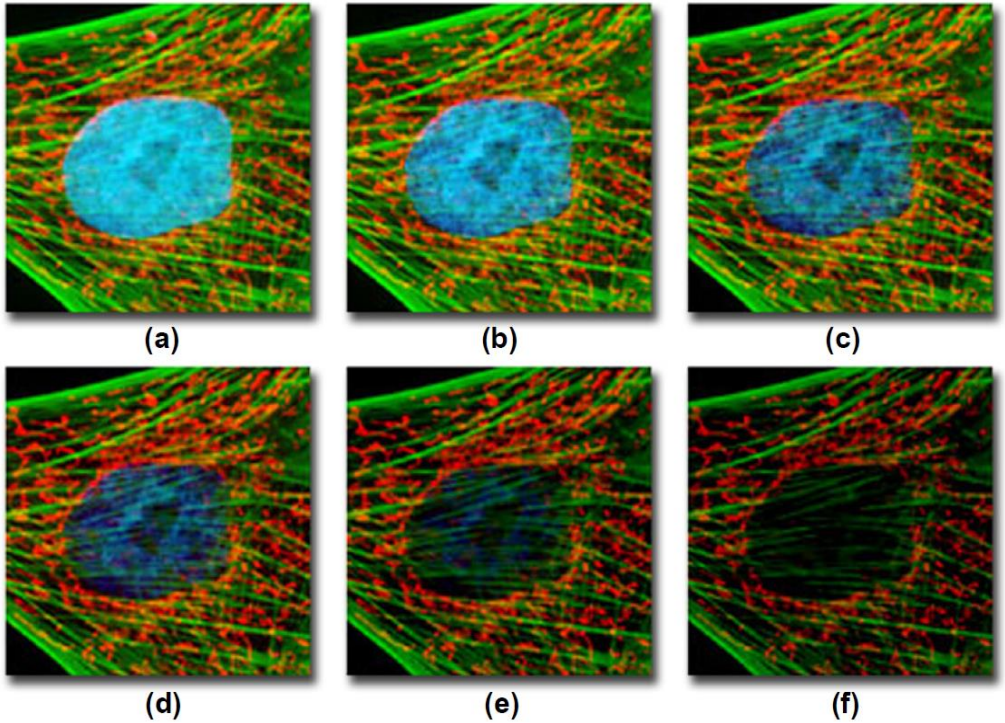


Figure 4 *- Photobleaching Rates in Multiply Stained Specimens*



Presented in Figure 4 is a typical example of photobleaching (fading) observed in a series of digital images captured at different time points for a multiply-stained culture of Indian Muntjac deer epidermis fibroblast cells. The nuclei were stained with a bis-benzimidazole derivative (Hoechst 33258; blue fluorescence), while the mitochondria and actin cytoskeleton were stained with MitoTracker Red CMXRos (red fluorescence) and a phalloidin derivative conjugated to Alexa Fluor 488 (green fluorescence), respectively. Time points were taken in

Figure 5 *- Arc-Discharge Fluorescence Lamps*

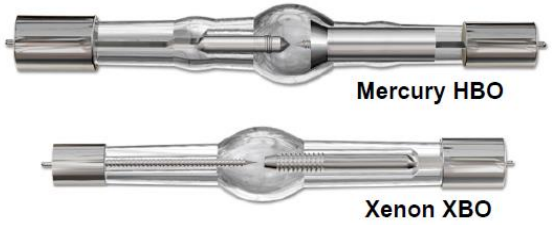
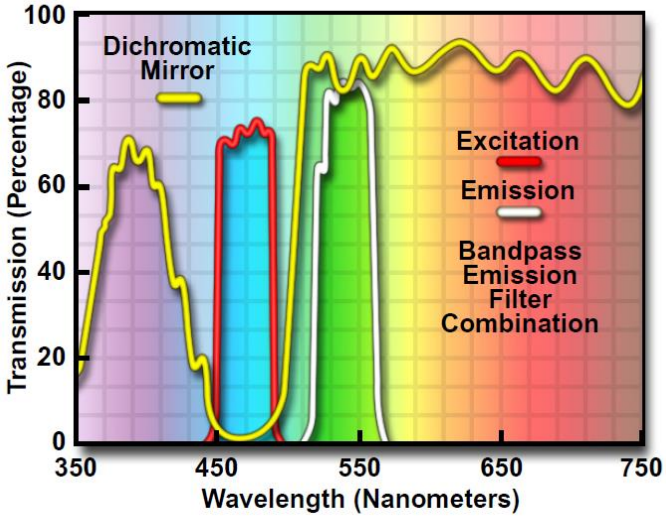
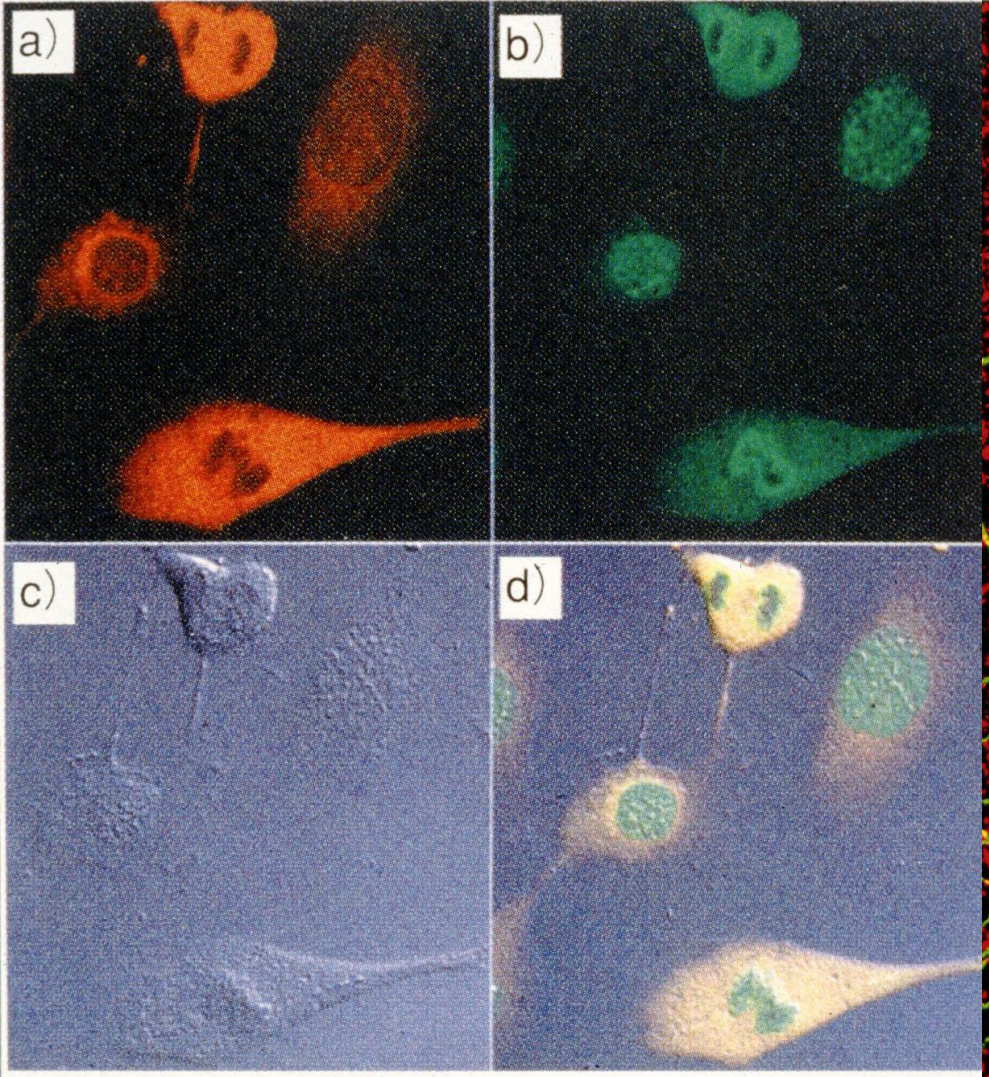
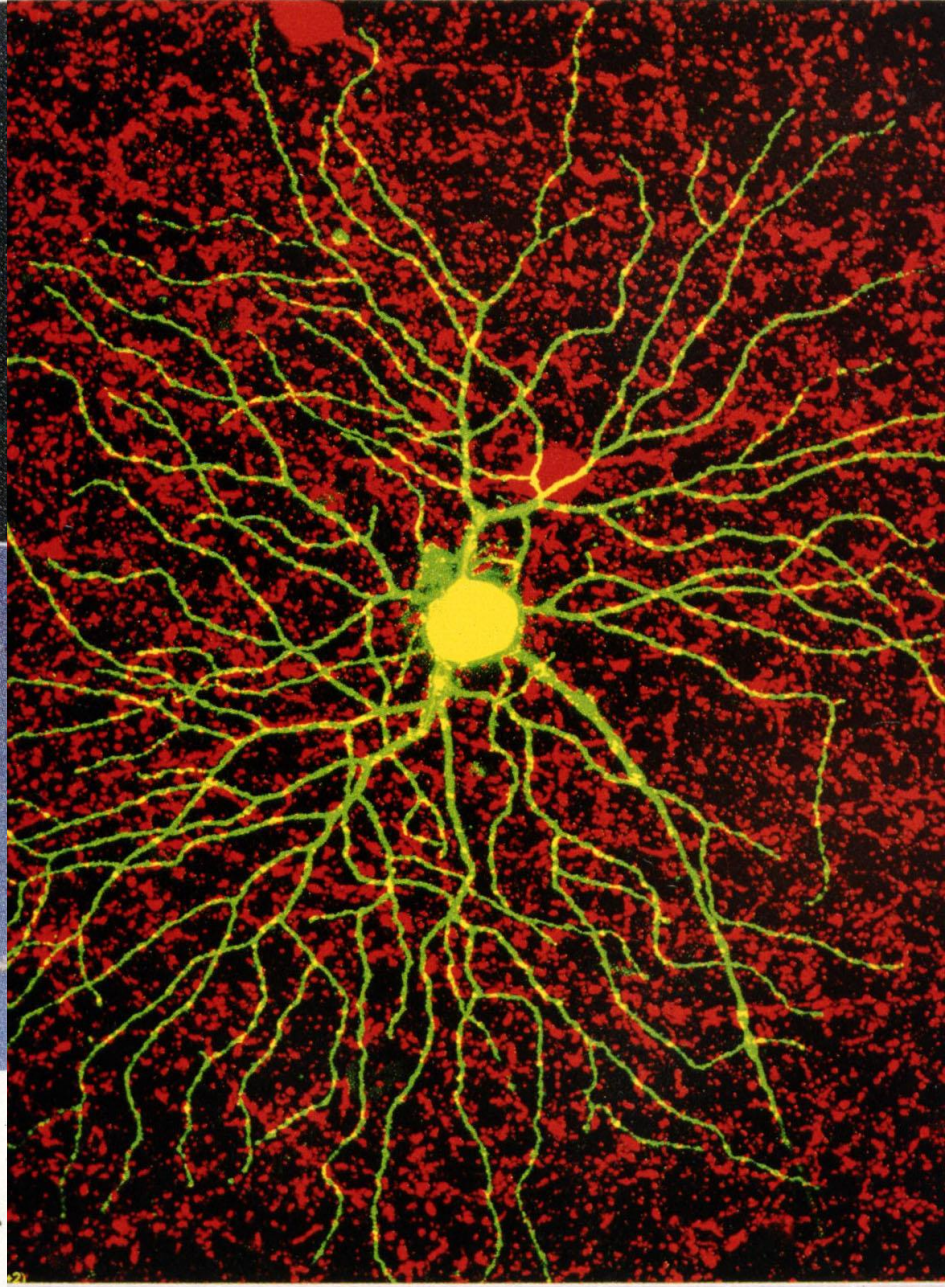


Figure 6 *- Nikon B-2E (Medium Band Blue Excitation)*

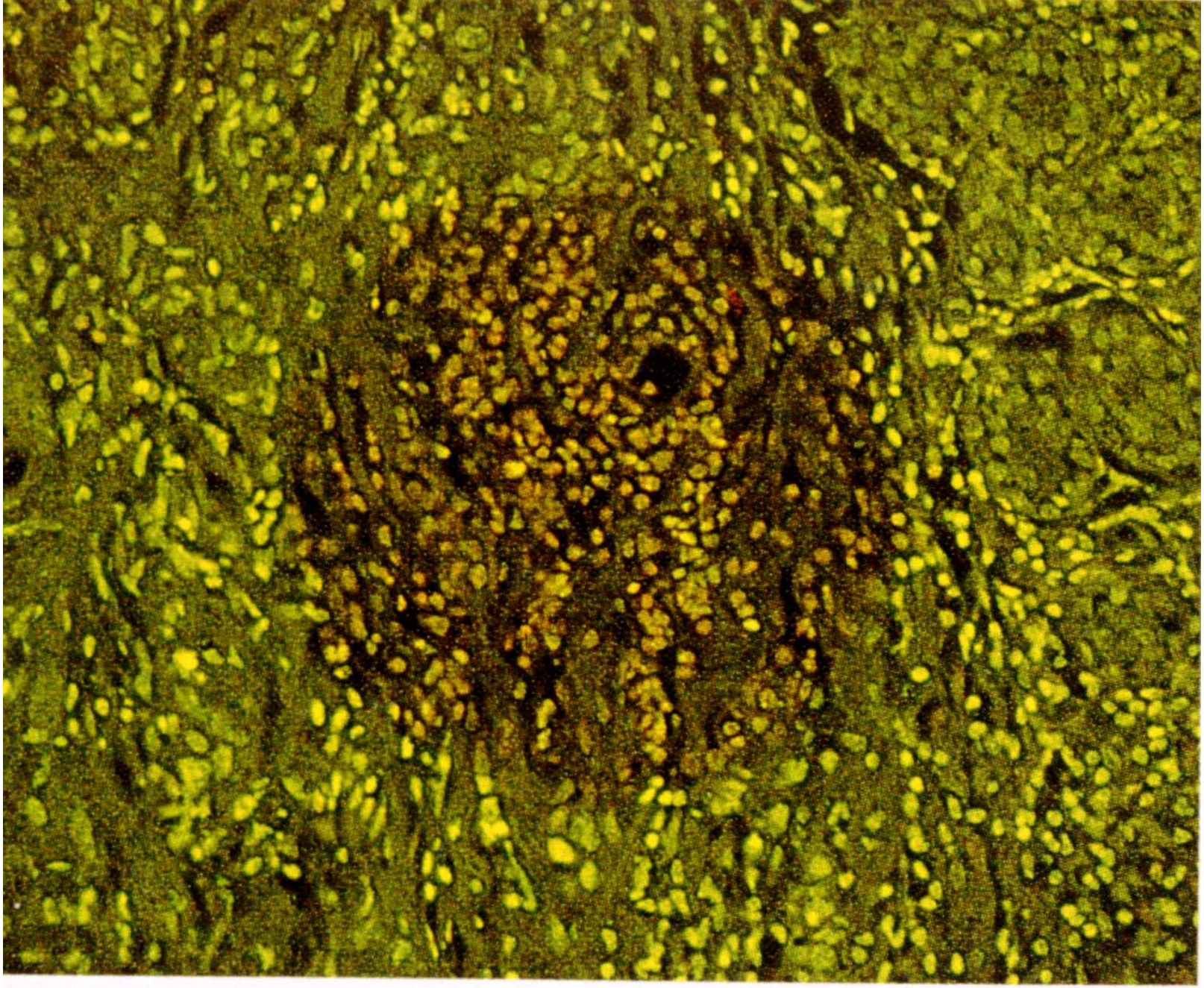




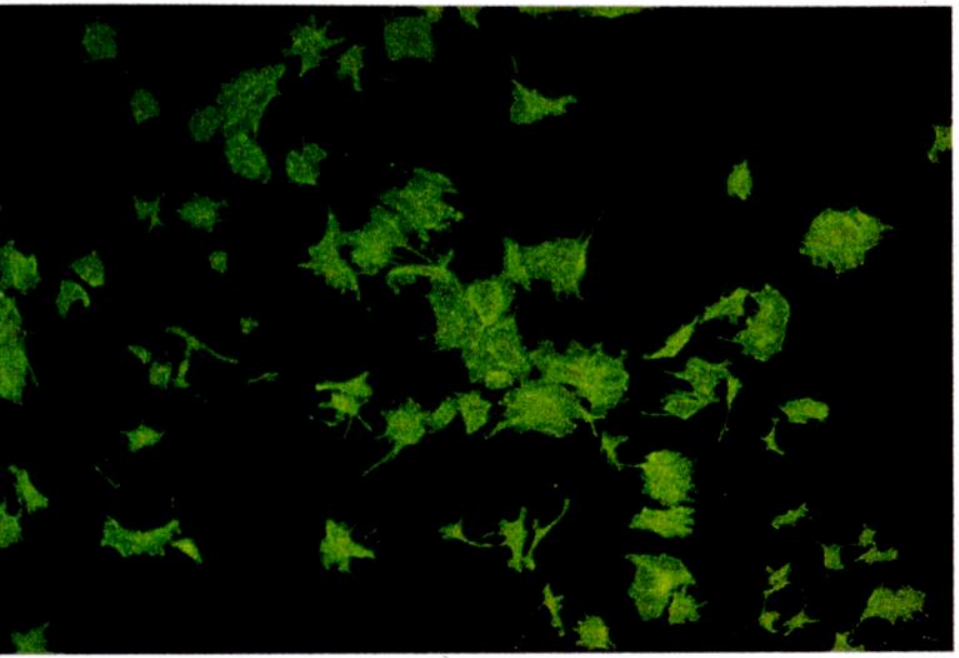
12　FITC，Rhodamine，微分干渉および合成像
a）F-アクチン/Rhodamine染色像，b）クリスン/FITC染色像，c）微分干渉像，d）合成像．
ラット由来の筋芽細胞（L-6）の観察像．
東京大学大学院総合文化研究科広域科学専攻生環境科学系跡見順子先生より提供



細胞とドーパミン作動性アマクリン細胞のレーザー顕微鏡エクス
像．Lucifer Yellowで網膜神経節細胞，Texas Redでドーパミン
を染色．



褪色してしまった例



B

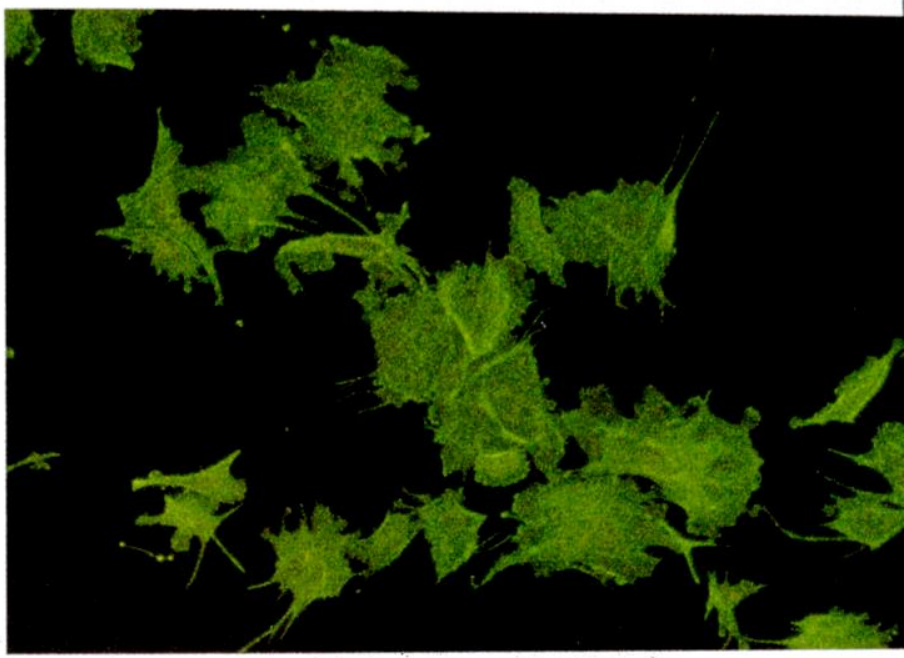
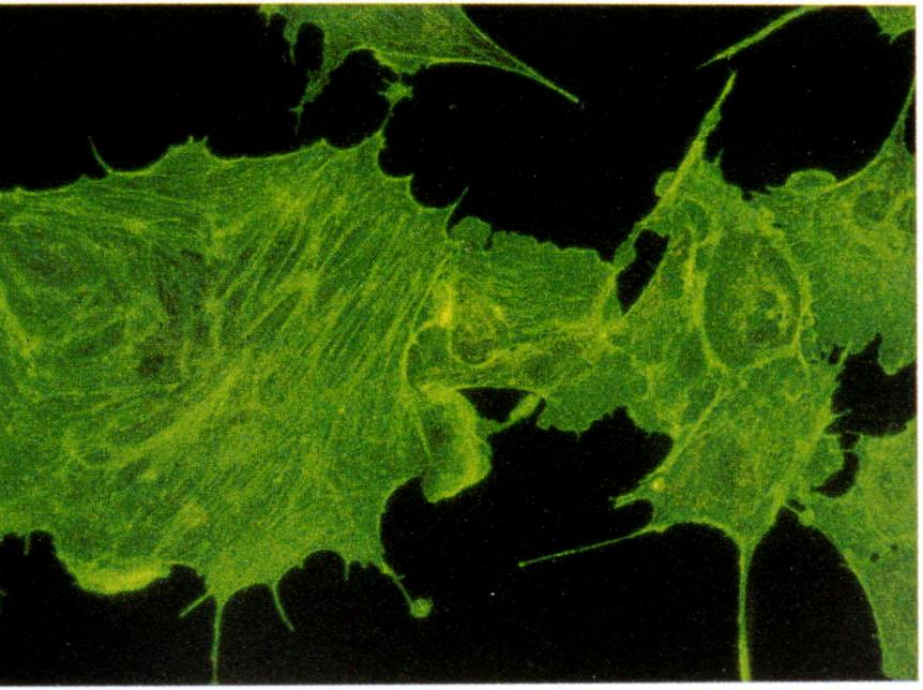


図5　倍率を切り換えたときの蛍光像

10×で観察した蛍光像（A，NDフィルターなし）と40×で観察したAの拡大像（B，ND25フィルターを使用）．A：低倍率で観察している場合は，NDフィルターを入れなくても適正な明るさの像が得られている．B：観察倍率を上げた場合には，ND25フィルターを入れて光量を落とさないと，適正な明るさにならない．



B

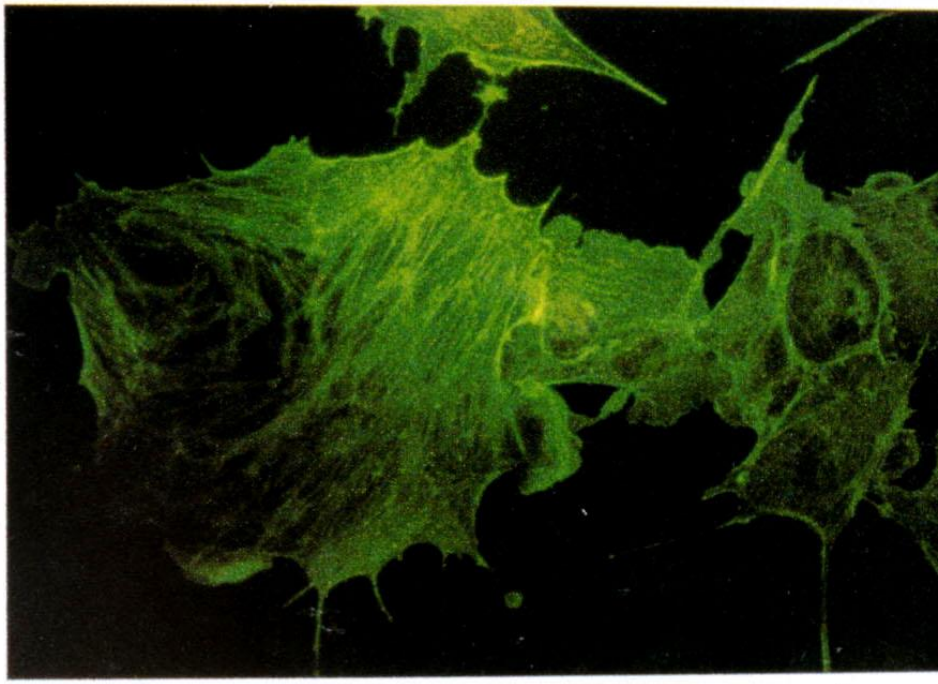


図7　水銀ランプの心出しの効果

水銀ランプの心出しを行った蛍光像（A）と行っていない蛍光像（B）．A：きちんと心出しが行われている場合は，視野全体が均一に照明されるため，蛍光像も均一な明るさになる．B：心出しが不十分な場合は，視野内が均一に照明されないため，蛍光像の中に明るい部分と暗い部分が生じてしまう．

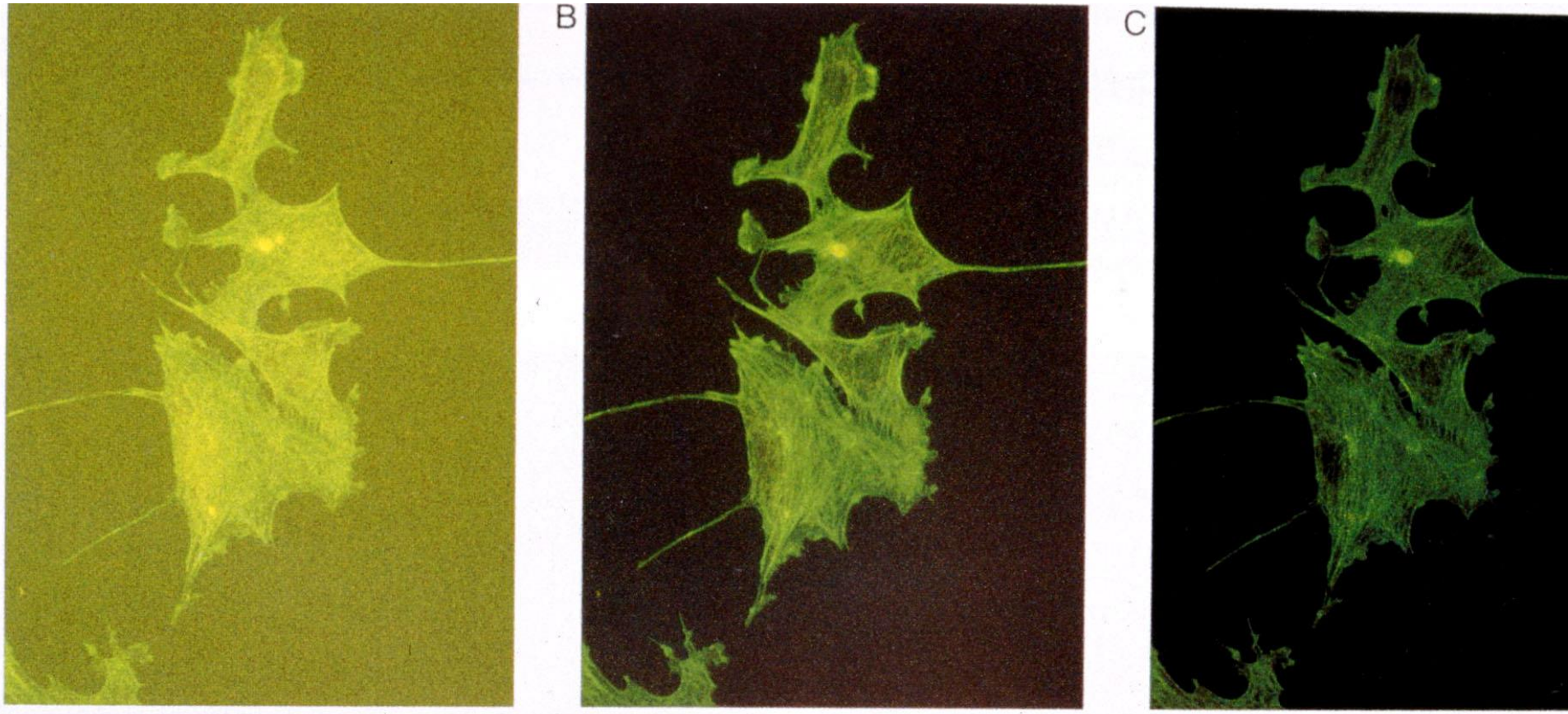


図9　励起フィルターの波長域の効果

A：蛍光キューブにU-MSWB2を使用したときのFITC蛍光像，励起フィルターの波長域が420～480nmと広いため，蛍光像は最も明るいが，同時にバックグラウンドも高くなっている．B：蛍光キューブにU-MWIB2を使用したときのFITC蛍光像，励起フィルターの波長域が460～490nmと中程度なので，蛍光像はほどほどに明るく，バックグラウンドもほどほどに出ている．C：蛍光キューブにU-MNIB2を使用したときのFITC蛍光像，励起フィルターの波長域が470～490nmと狭いため，蛍光像は最も暗いが，同時にバックグラウンドも低くなっている．これらのバックグラウンドは，主に対物レンズと培地の自家蛍光によるものである．

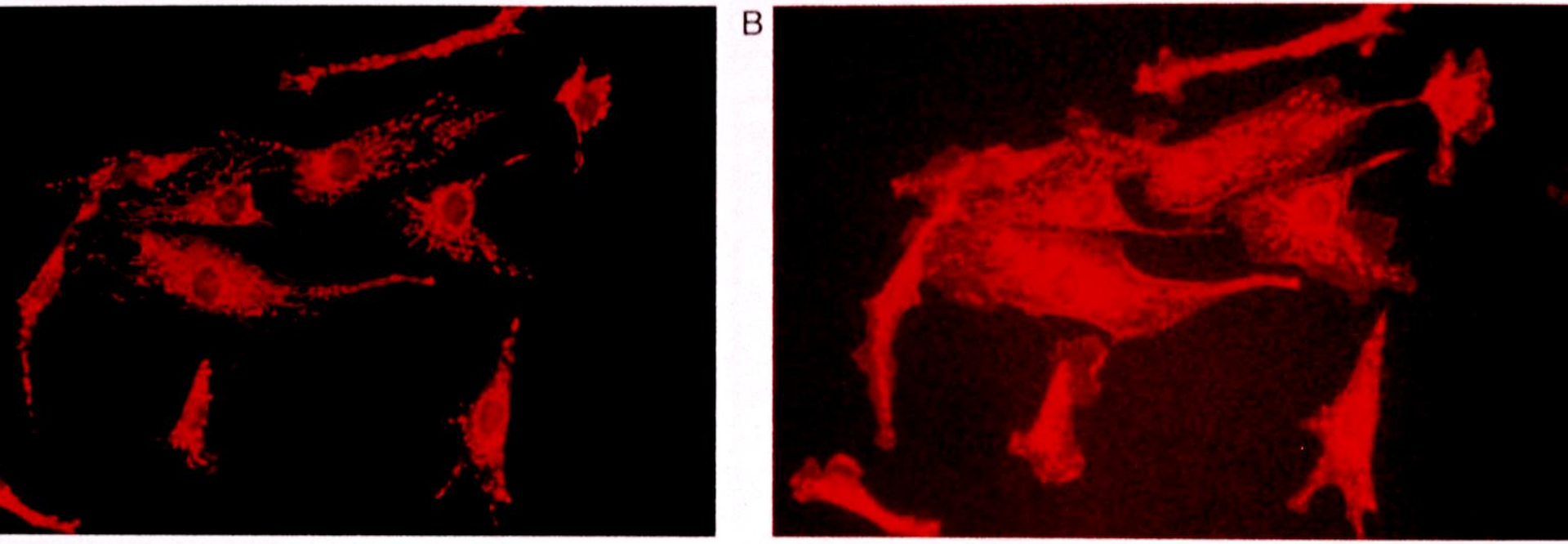
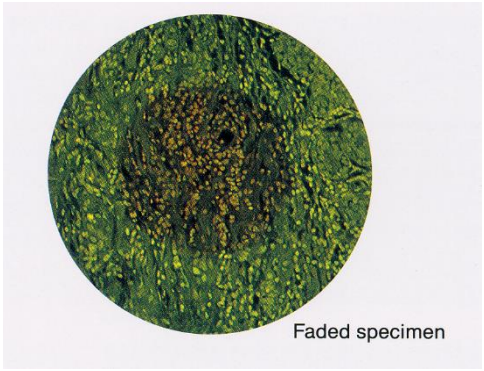


図4　励起光の強さの効果

適切な強さの励起光が当たっている蛍光像（A）と励起光が強すぎる蛍光像（B）．A：励起光の強度が適切な場合は，バックグラウンドが暗黒に近くなり，蛍光像を高いコントラストで観察できる．また，褪色も抑えられる．B：励起光が強すぎると，蛍光像は明るいが，同時にバックグラウンドも明るく，コントラストは劣ってしまう．標本の褪色も早い．

常見的抗退劑

- P-phenylenediamine
- DABCO
- N-propylgallate
- 2-mercaptoethylamine



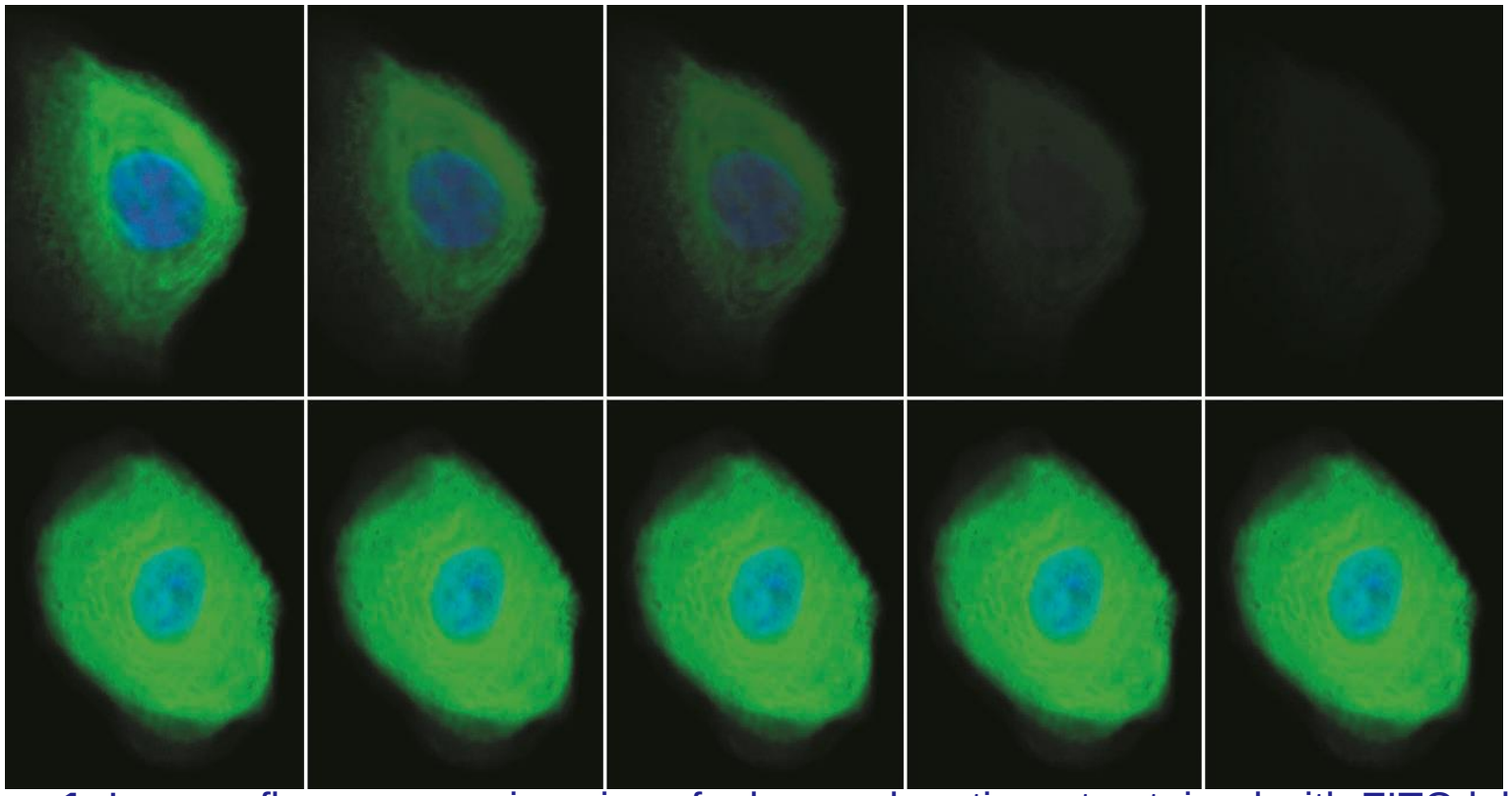
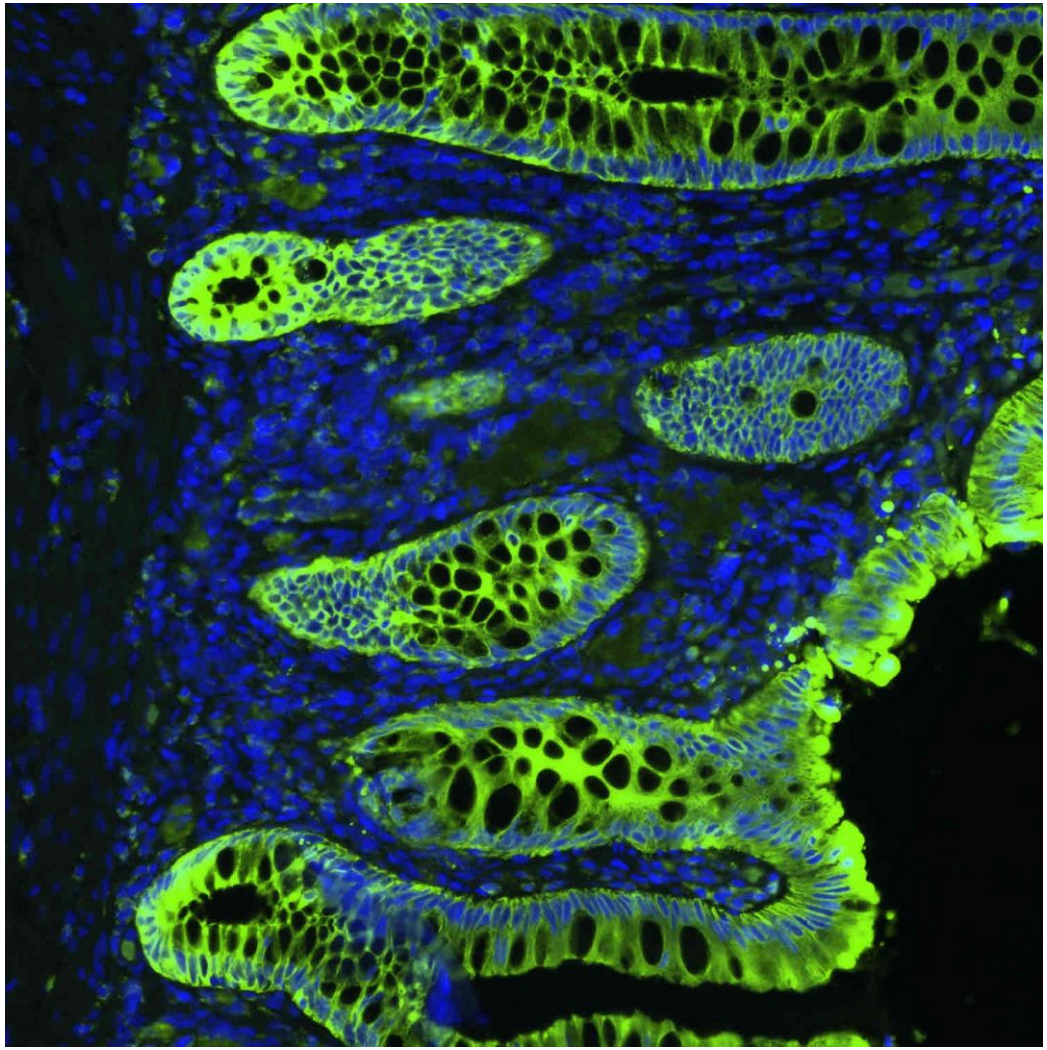


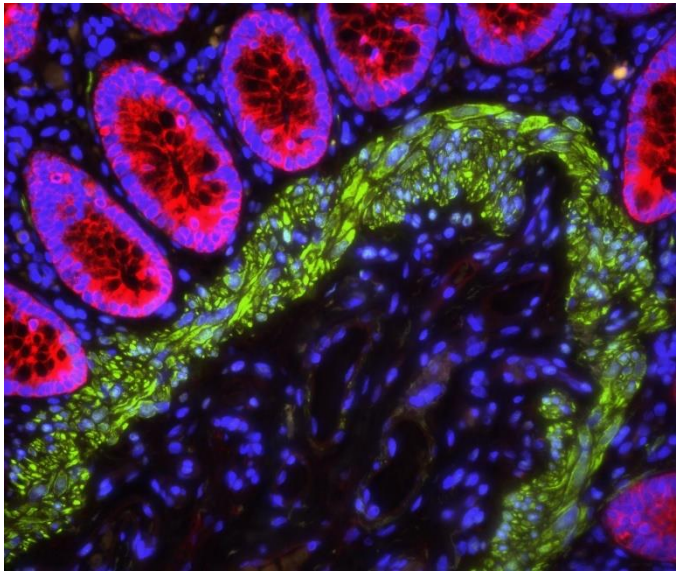
Figure 1. Immunofluorescence imaging of a human keratinocyte stained with FITC-labeled antibodies without (top) and with (bottom) PromoFluor Antifade Reagent at different excitations intervals (5, 10, 15, 20, 25 seconds). **PromoFluor Antifade Reagent**

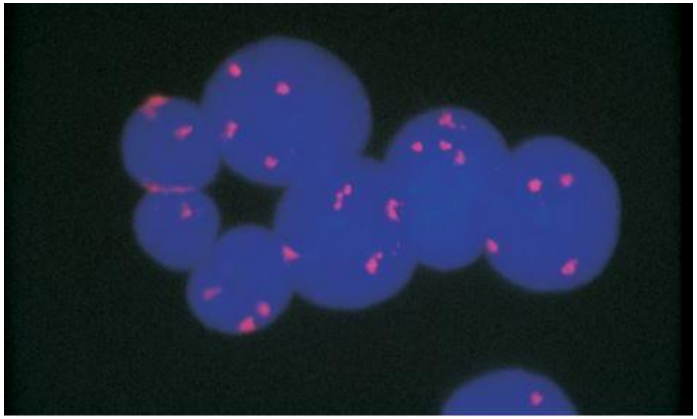
VECTASHIELD® Vibrnce Antifade Mounting Medium

VECTASHIELD® PLUS Antifade Mounting Medium with DAPI

from Vector Laboratories








Thank you for your attention