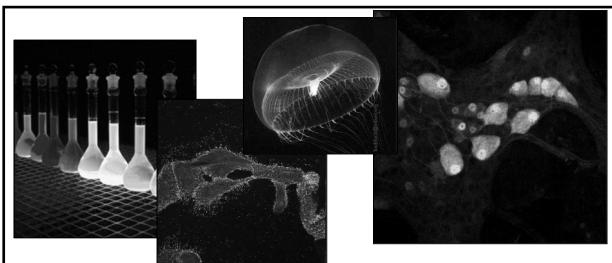


Microscopische technieken



Inleiding microscopische technieken en weefselvoorbereiding

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1

Microscopische technieken

LEERDOELEN

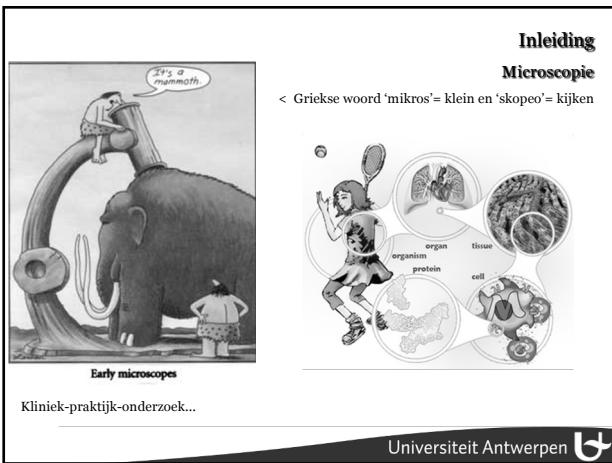
- Lichtmicroscopische technieken kennen
- Elektronenmicroscopische technieken kennen
- (Geavanceerde microscopische technieken worden verder belicht in het vak 'Biomedische beeldvorming' in BMW3)
- Beelden van coupes en microscopische technieken koppelen
- Verschillen tussen LM en EM preparatietechnieken verklaren
- Bewust worden van grootteverschillen in cellen en weefsels en het bereik van de gehanteerde microscopische technieken

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2

Inleiding Microscopie

< Griekse woord 'mikros'= klein en 'skopeo'= kijken



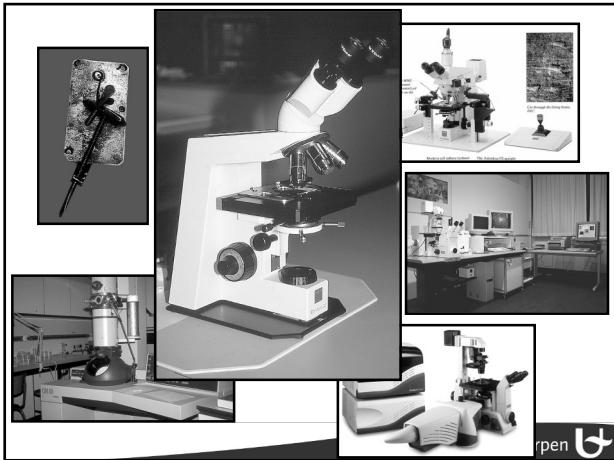
Early microscopes

Kliniek-praktijk-onderzoek...

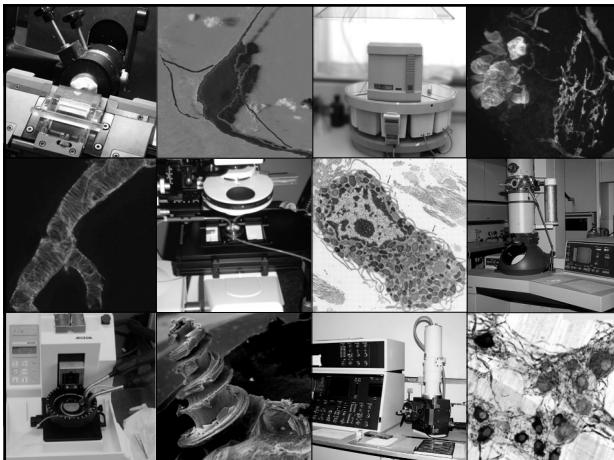
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3

Microscopische technieken



4



5

Hoe groot is een gemiddelde cel ?

0.1	1	10	100	1	10	100	1	1	10
nm					μm				

6

Microscopische technieken

When poll is active, respond at pollev.com/cbhacam
Text CBHACAM to +32 460 20 00 56 once to join

hoe groot is een gemiddelde cel?

- A : 2 micrometer
- B: 0,2 micrometer
- C: 0,5 millimeter
- D: 10 micrometer
- E: 10 nanometer
- F: 100 micrometer

Start the presentation to see live content. For screen share software, share the entire screen. Get help at pollev.com/app

7

When poll is active, respond at pollev.com/cbhacam
Text CBHACAM to +32 460 20 00 56 once to join

wat is de gemiddelde doormeter van een normale menselijke rode bloedcel of erytrocyt?

- A: 6 micrometer
- B: 6 nanometer
- C: 60 nanometer
- D: 100 micrometer
- E: geen van bovenstaande

Start the presentation to see live content. For screen share software, share the entire screen. Get help at pollev.com/app

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0.1	1	10	100	1	10	100	1	1	10
nm			μm				mm	cm	

Eicel
Cel
RBC

Eicel – follikel
Zenuwcel
Spiercellen (lengte tot enkele tientallen cm)

9

Microscopische technieken

Welke celorganellen ken je ?

0.1	1	10	100	1	10	100	1	1	10
nm		μm			mm		cm		

Zie ook cursus ‘Biomoleculen en cellen’ !!!!!

10

welke celorganellen ken je?

Start the presentation to see live content. For screen share software, share the entire screen. Get help at pollev.com/app

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When poll is active, respond at pollev.com/cbhacam
Text **CBHACAM** to +32 460 20 00 56 once to join

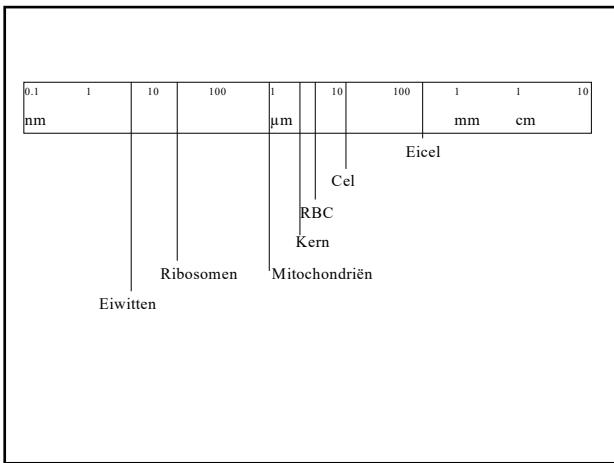
hoe groot is een mitochondrium op dwarse doorsnede?

- A: 0,2 millimeter
- B: 2 nanometer
- C: 2 micrometer
- D: 0,2 micrometer
- E: geen van bovengenoemde

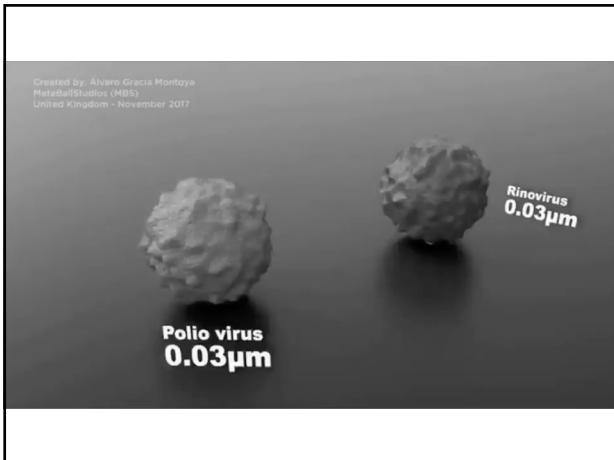
Start the presentation to see live content. For screen share software, share the entire screen. Get help at pollev.com/app

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Microscopische technieken



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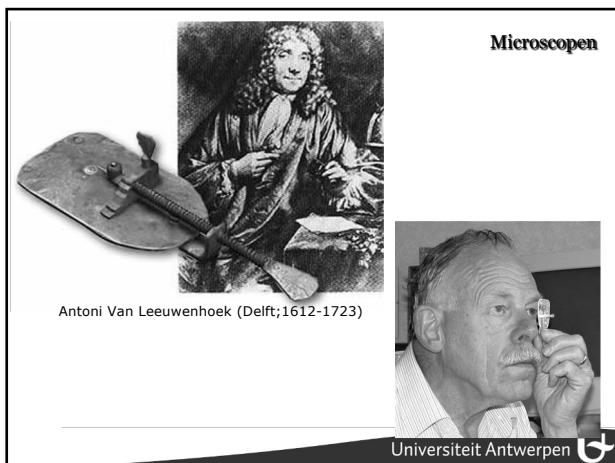


14

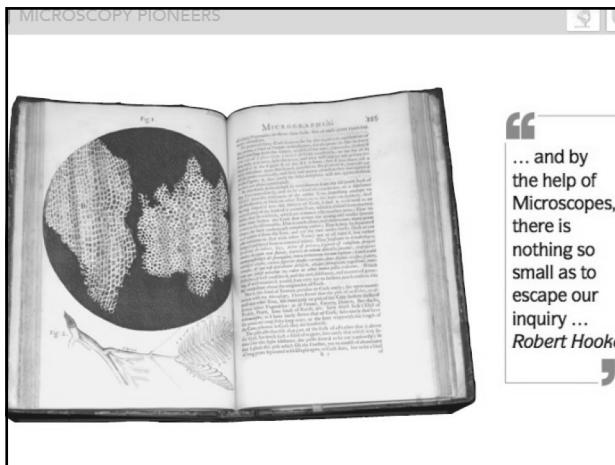


15

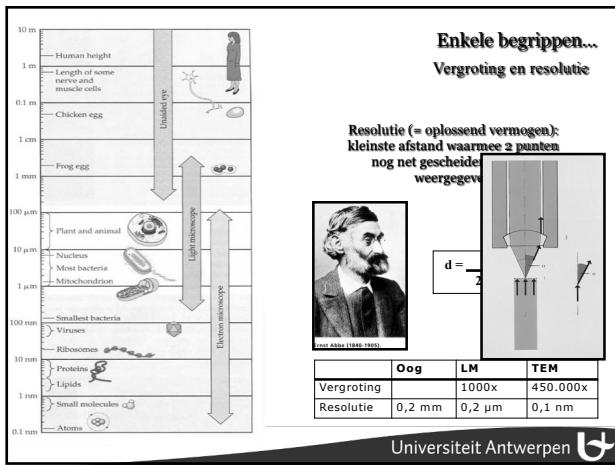
Microscopische technieken



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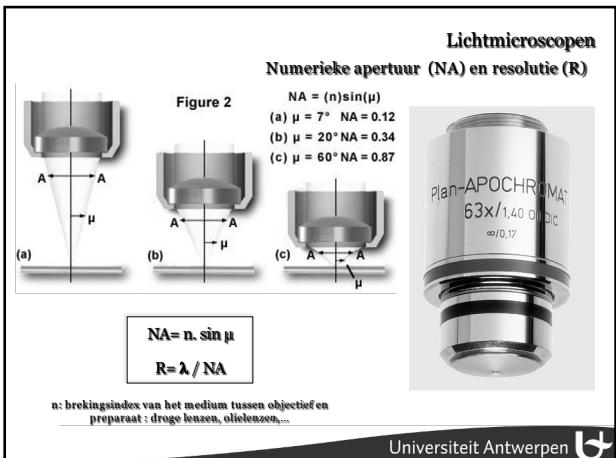


17

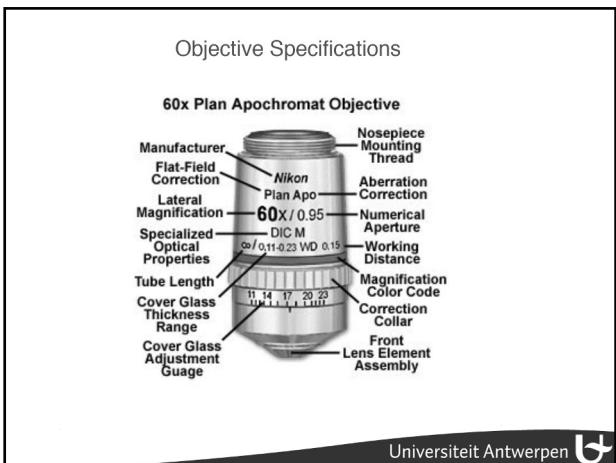


18

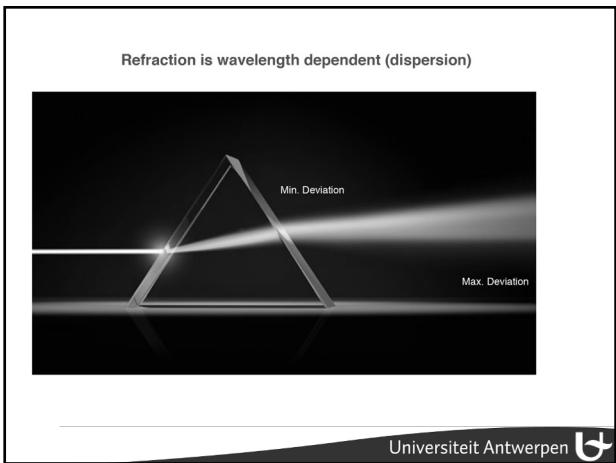
Microscopische technieken



19

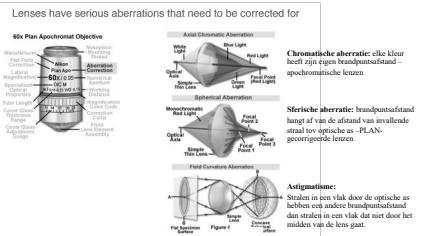


20



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Lensfouten

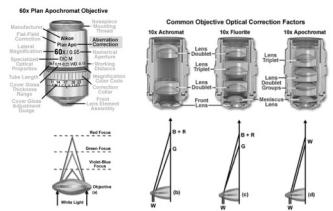


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Lensfouten (2)

Lenses have serious aberrations that need to be corrected for



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Resolutie

$$R = (K \times \lambda) / NA$$

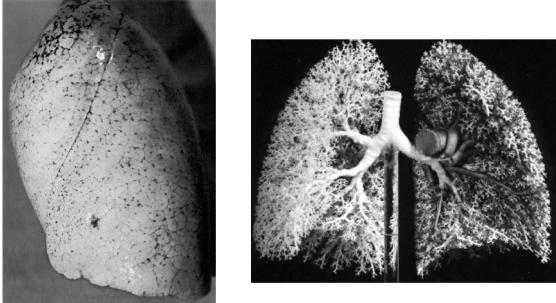
Lichtmicroscoop	Elektronenmicroscoop
$\lambda = 550 \text{ nm}$	$\lambda = 0.005 \text{ nm}$
Res-LM = $0,2 \mu\text{m}$	Res-EM = $0,2 \text{ nm}$
Minot-microtoom	Ultramicrotome
Coupedikte: $5 \mu\text{m}$	Coupedikte: 50 nm
Kleuring	Contrastering
Kleurstoffen	Zware metalen

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Microscopische technieken

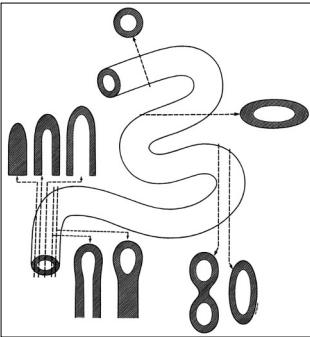
Probleem...



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Lichtmicroscopen
Preparatie van paraffinecoupes



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Weefselvoorbereiding...
Fixatie

Moet zo snel mogelijk gebeuren !!
Kan door immersie (onderdompelen) of perfusie (via bloedbaan)



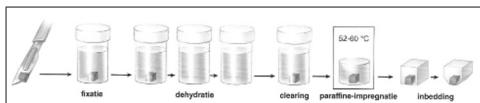
Chemische fixatieve: Paraformaldehyde, glutaraldehyde
= bewaren van ultrastructuur van weefsels en cellen d.m.v. cross-linking van eiwitten

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Microscopische technieken

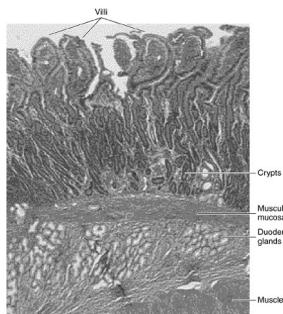
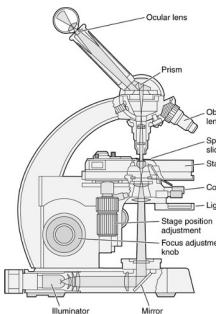
Weefselvoorbereiding Van orgaan naar coupe (1)



Fixatie
Inbedding
Snijden en opvangen
Kleuren

28

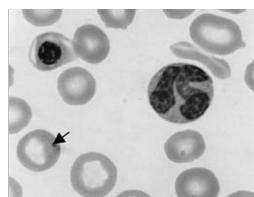
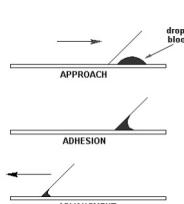
Helderveld lichtmicroscoop



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Weefselvoorbereiding...

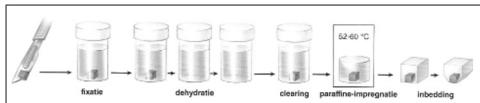
... is sterk afhankelijk of je levende cellen wil visualiseren, volledige stukjes van organen, perfect 'bewaarde' cellen en weefsels,...
... is sterk afhankelijk van de microscoop die je gebruikt.



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Microscopische technieken

Weefselvoorbereiding Van orgaan naar coupe (1)



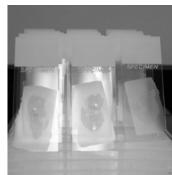
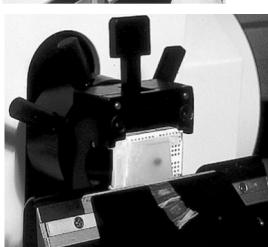
Fixatie
Inbedding
Snijden en opvangen
Kleuren

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Lichtmicroscopen

Preparatie van paraffinecoupes

Orgaan fixeren en naspoelen – ontwateren (stijgende alcoholreeks) – inbedden in paraffine – microtomecoupes (ong. 5 µm dik) – opvangen op draagglasje en drogen



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Lichtmicroscopen

Preparatie van paraffinecoupes

Kleuring	Samenstelling	Kern	Cytoplasma	Collagene vezels	Elastine vezels
HE	Haematoxyline en eosine	Purper	-	Roze	-
AZAN	Asocarmijn Anilineblauw Oranje G	Rood	Rood	-	-
Trichroom	Ijzerhaematoxyline Zure fuchsinine, Ponceau R Lichtgroen of anilineblauw of heidenhansblauw	Zwart	-	-	-
Orcelina	Orcelina	-	-	-	roodbruin

Rehydrateren (dalende alcoholreeks) – kleuren – stijgende alcoholreeks- insluiten

Kleuren : vb haematoxyline (basische kleurstof) bindt aan zuren (vb nucleinezuren)
eosine (zure kleurstof) bindt aan basische stoffen (vb eiwitten)

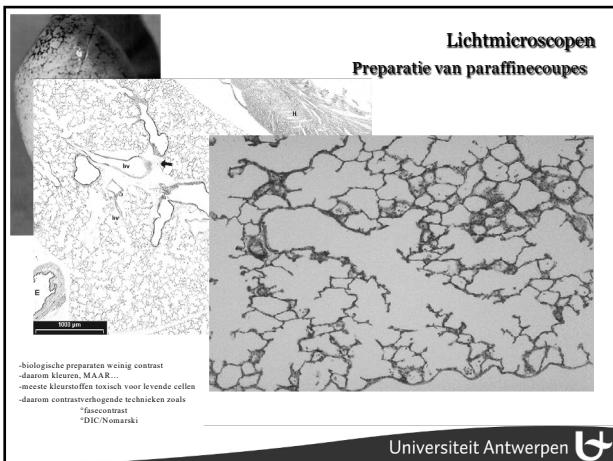
'Histochemie': vb Periodic Acid Schiff (PAS) reactie voor aantonen glycogeen

=> Verdere specifieke kleuringen: zie practicum

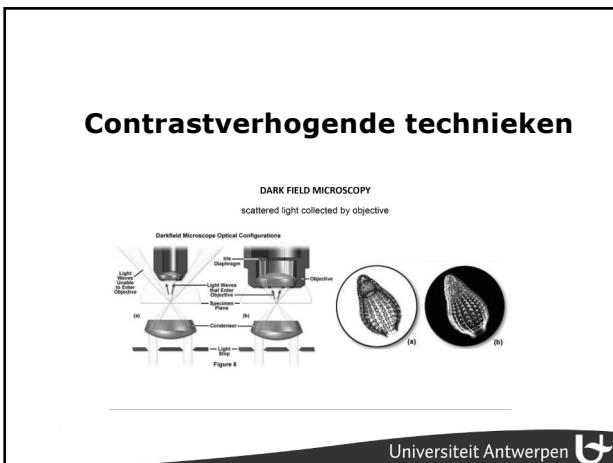
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Microscopische technieken



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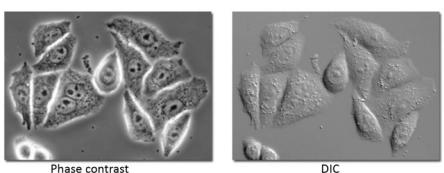


35



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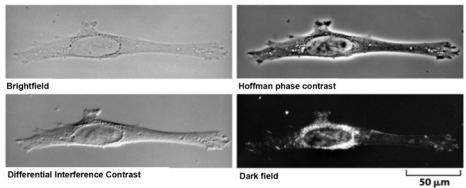
Contrastverhogende technieken



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Contrastverhogende technieken



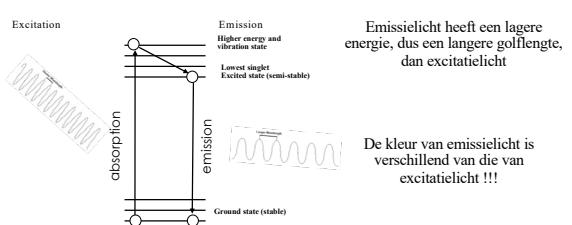
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Wat is fluorescentie? Principe...

Fluorescentie is de eigenschap van bepaalde atomen en moleculen om licht van een bepaalde golflengte te absorberen en na een korte tijd (= fluorescence lifetime) terug uit te zenden met een langere golflengte.

Fluorescence



Fluorochromen

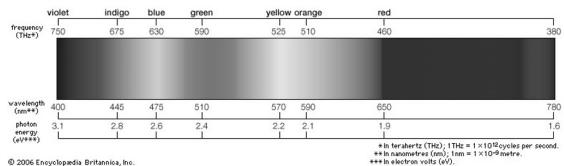
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Microscopische technieken

Wat is fluorescentie?

Zichtbaar spectrum van het licht: 400 nm – 700 nm



© 2006 Encyclopædia Britannica, Inc.

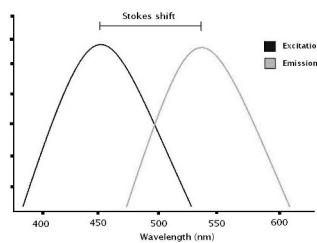
Korte golflengten: hoge frequentie, hoge energie
Lange golflengten: lage frequentie, lage energie

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Wat is fluorescentie?

Excitation, Emission and Stokes shift



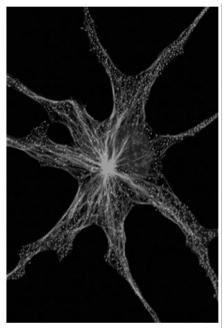
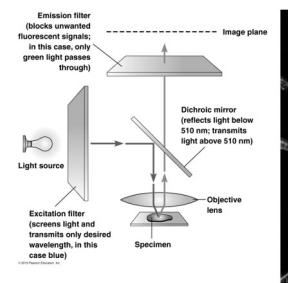
Een fluorofoon heeft een excitatie en emissiespectrum

Het verschil tussen de maxima van absorptie en emissie spectra noemt men de **Stokes shift**

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Fluorescentiemicroscopie



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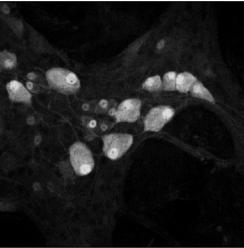
42

Microscopische technieken

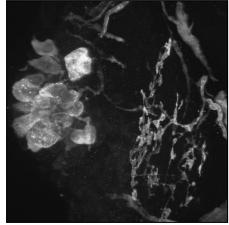
Lichtmicroscopie: Fluorescentiemicroscopie

Wat is fluorescentie?

Fluorescente kleuringen en kleurstoffen worden gebruikt om structuren en processen in biologische samples te detecteren en visualiseren.



Van Nassauw et al.
Histochem. Cell Biol, 2005



Brouns et al.
Histochem. Cell Biol, 2006

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De fluorescentiemicroscoop...

Fluorochromen

✗ Autofluorescentie

Planten: bv. pollenkorrels, chloroplasten
Dieren: bv. Bepaalde pigmenten (haar),

✗ Immunohistochemische kleuringen: antilichamen !!
Traditionele fluorescente kleurstoffen : FITC, DAPI, Hoechst dyes...
Cyanine dyes: Cy3, Cy5, Cy7,...
Alexa Fluor dyes: Alexa Fluor 488, Alexa Fluor 568,...
Quantum dots

✗ Levende organismen
Fluorescent functionele : Fluo-4, Fura Red
Organel Probes: MitoTracker, Lysotracker
Fluorescente eiwitten: GFP, YFP

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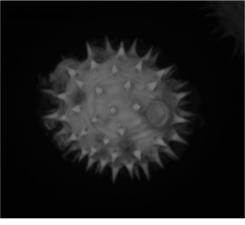
44

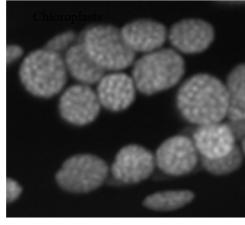
De fluorescentiemicroscoop...

Fluorochromen

✗ Autofluorescentie

Planten: pollen korrels, chloroplast





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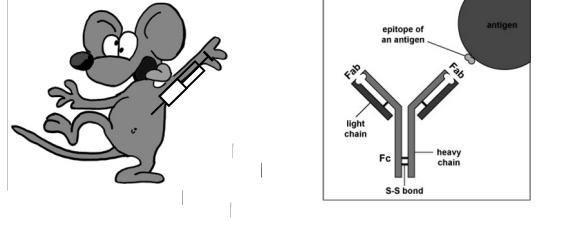
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Microscopische technieken

De fluorescentiemicroscoop...
Fluorochromen

*Immunohistochemische kleuringen (immunofluorescentie):
verbonden met antilichamen !!!

... antilichamen worden 'aangemaakt' in een dier tegen een bepaald 'epitool' van
een eiwit en daarna geïsoleerd.



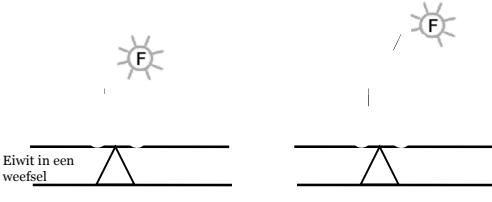
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De fluorescentiemicroscoop...
Fluorochromen

*Immunohistochemische kleuringen (immunofluorescentie):
verbonden met antilichamen !!!

... wanneer ze op weefsels/cellen worden gebracht zullen deze antilichamen
binden aan het eiwit waartegen ze zijn opgewekt



Eiwit in een weefsel

Directe procedure

indirecte procedure

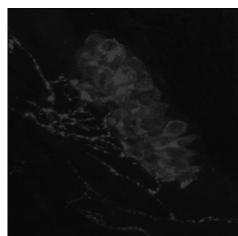
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De fluorescentiemicroscoop...
Fluorochromen

*Immunohistochemische kleuringen (immunofluorescentie):
verbonden met antilichamen !!!

... wanneer antilichamen fluorochromen zijn verbonden is het mogelijk deze
te zien in een fluorescentiemicroscoop.



Longsnede van een rat,
immunofluorescentie met antilichamen
opgewekt in konijn tegen CGRP,
Cy3 fluorochroom

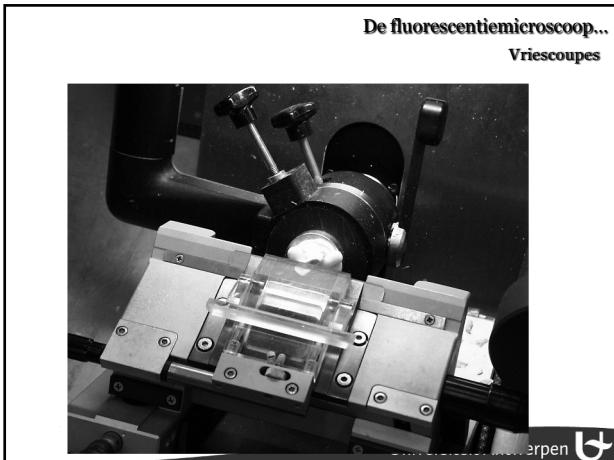
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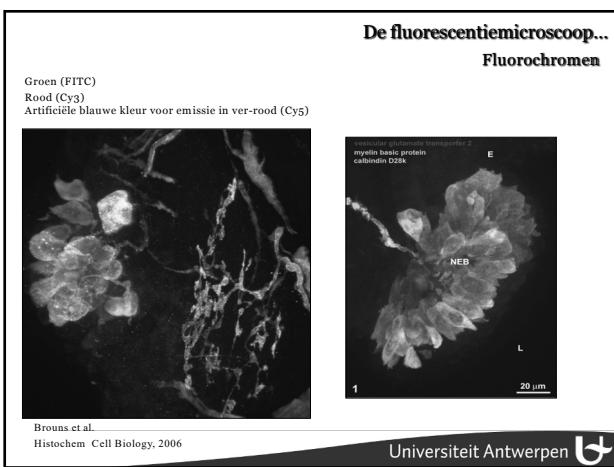
Microscopische technieken



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Microscopische technieken

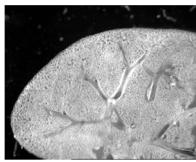
De fluorescentiemicroscoop... Fluorochromen

* Levende organismen

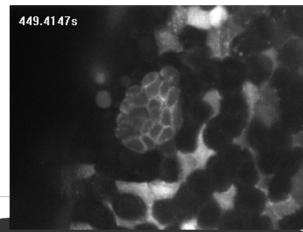
Fluorescente functionele probes: e.g. Fluo-4, Fura Red

... fluoroforen die veranderingen in de levende cel kunnen visualiseren
(cell culturen/ex vivo weefsels)

... detecteren veranderingen in ionen-concentraties(Ca^{2+} , NO_x), pH, ...



Luchtwegepitheel in een ex vivo long slice
Gedoten met Fluo-4 (calciumindicator)



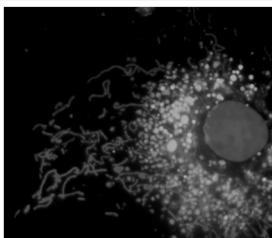
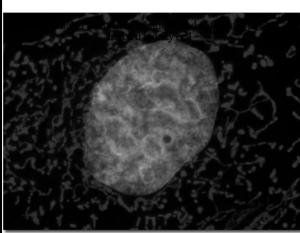
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Fluorophores/Fluorochromes...

* Living organisms

OrganelProbes: MitoTracker, LysoTracker

... fluoroforen 'targeten' intracellulaire organelen, e.g. Mitochondria, Golgi
apparatus, endoplasmatisch reticulum



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Fluorophores/Fluorochromes...

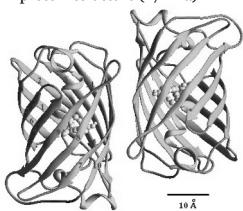
* Living organisms

Fluorescente eiwitten: GFP, YFP, DsRed

... natuurlijk voorkomende fluorescente eiwitten en hun analogen

... green fluorescent protein (GFP)

GFP protein structure (27 kDa)



reporter gene

gene N

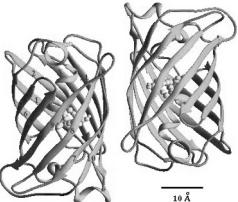
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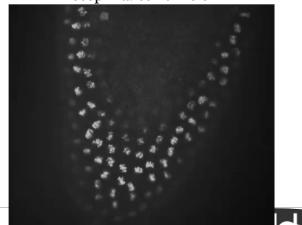
Fluorophores/Fluorochromes...

Living organisms
Fluorescente eiwitten: GFP, YFP, DsRed
... natuurlijk voorkomende fluorescente eiwitten en hun analogen
... green fluorescent protein (GFP)

GFP protein structure (27 kDa)

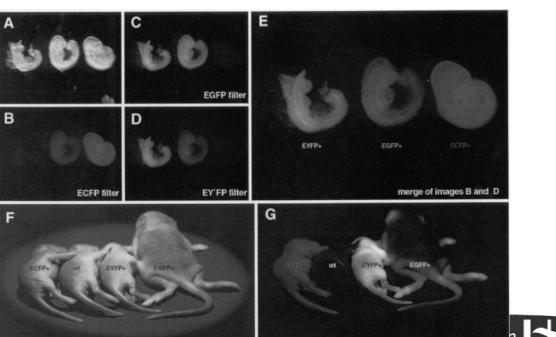


Drosophila: cell division

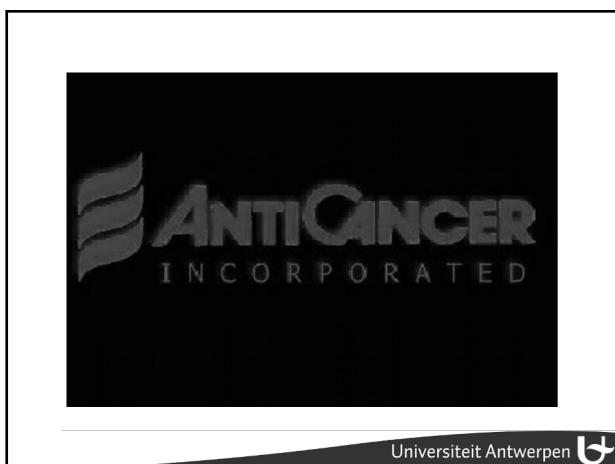


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Toepassing
→ gemodificeerd materiaal herkennen
in verschillende stadia



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Microscopische technieken

Confocal Laser Scanning Microscopy

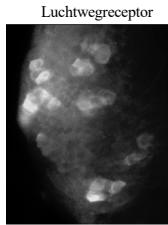
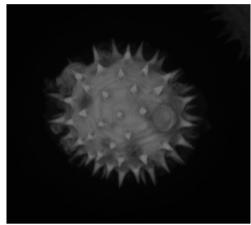


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Confocale microscopie

Out-of-focus blur in epifluorescentie



Bij gewone fluorescentiemicroscopie wordt het beeld gevormd door emissielicht afkomstig van de volledige dikte van het preparaat => out-of-focus 'blur' (wazig beeld)

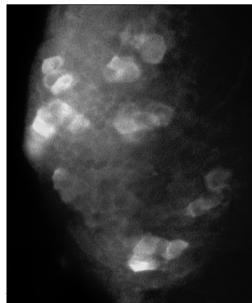
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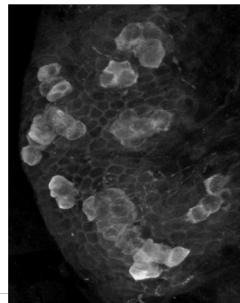
Confocal Laser Scanning Microscopy

Optical sections

Widefield epi-fluorescence



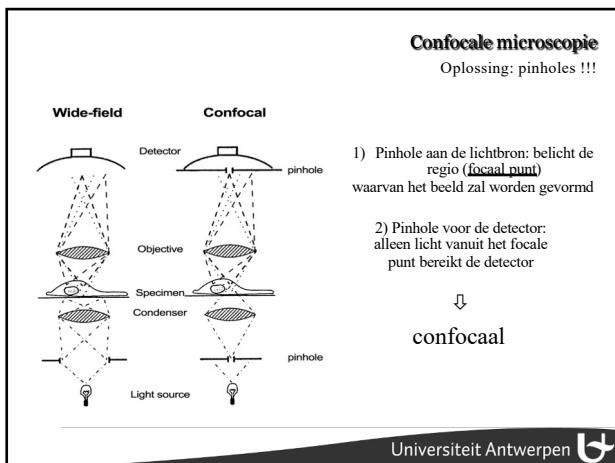
Confocal Maximal intensity projection -2D



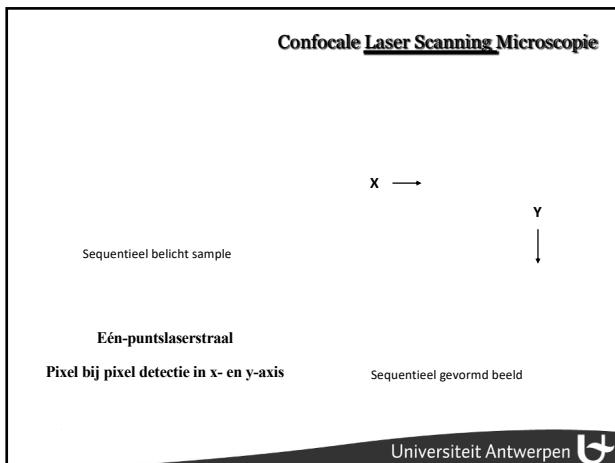
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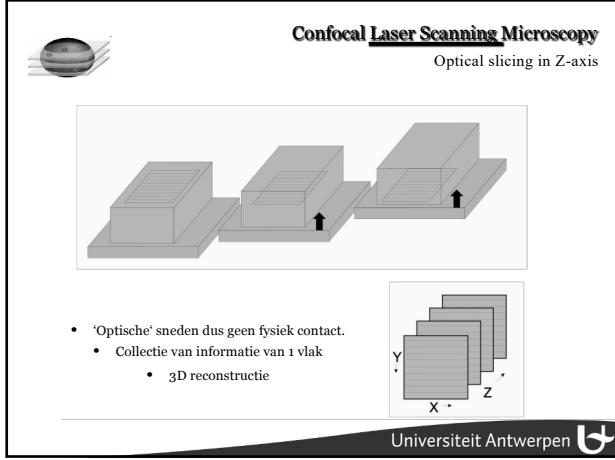
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Microscopische technieken



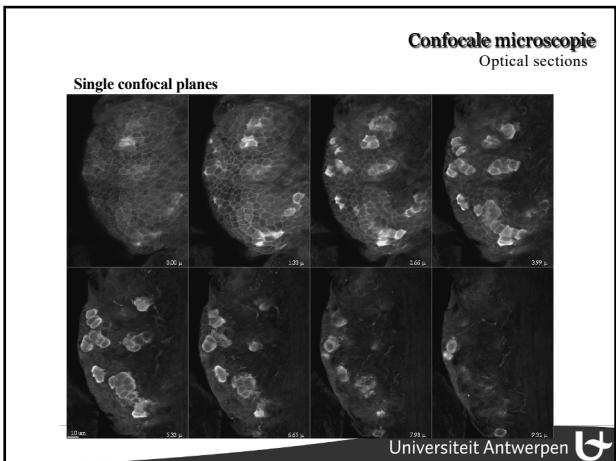
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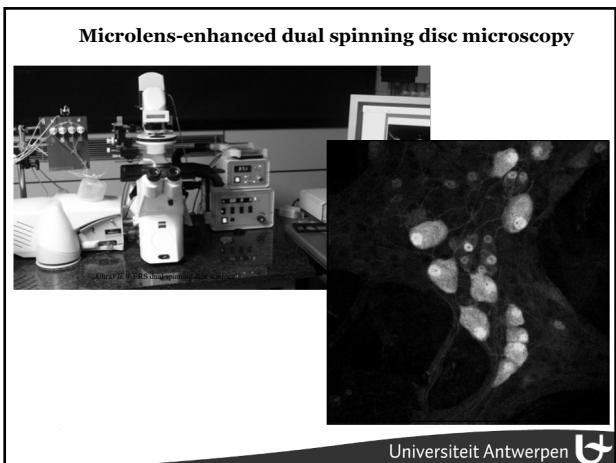


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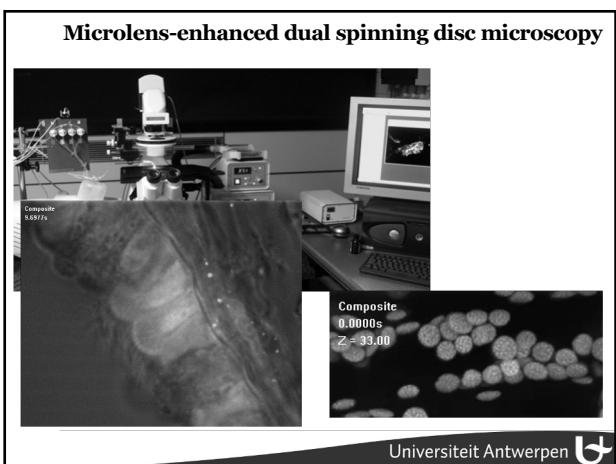
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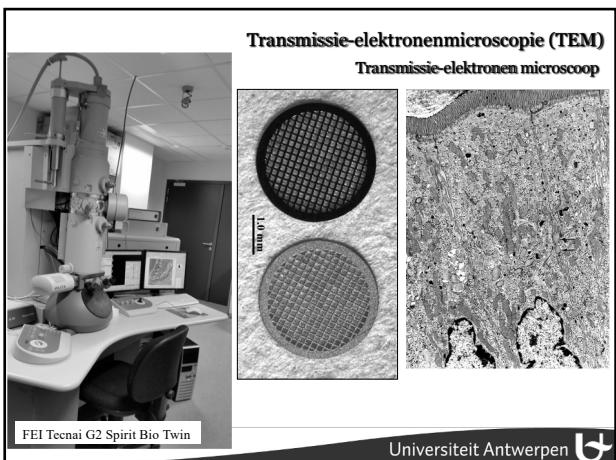


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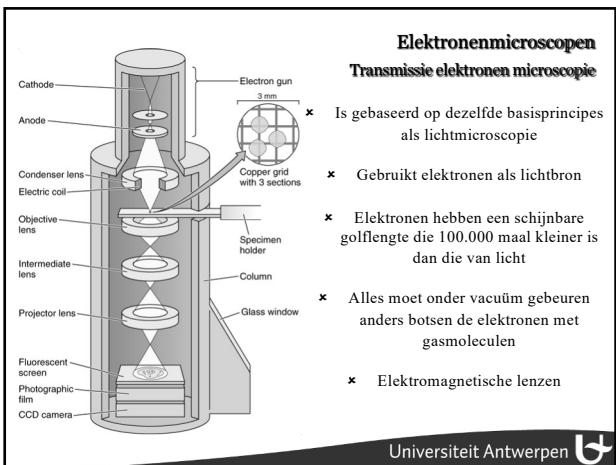


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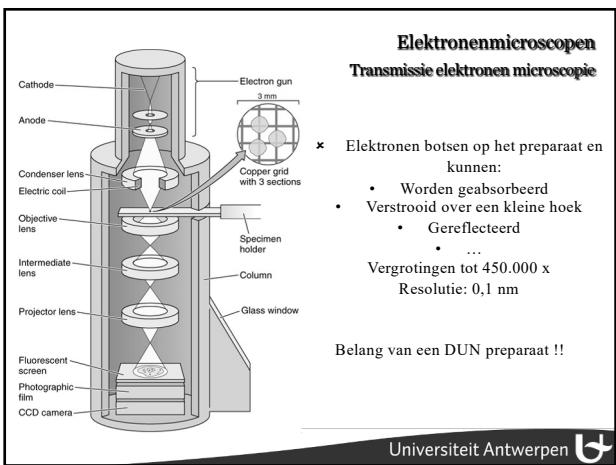
Microscopische technieken



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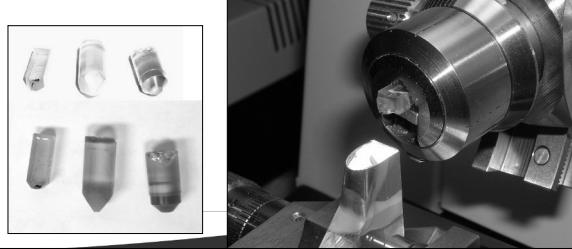


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Microscopische technieken

Maken van preparaten voor elektronenmicroscopie
Transmissie elektronen microscopie

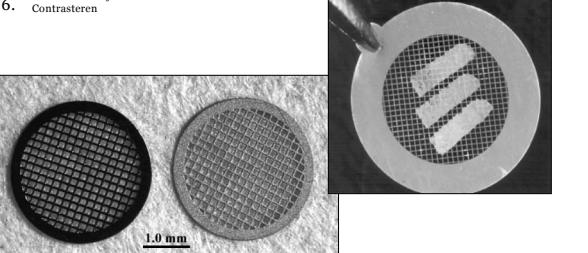
1. Fixatie: immersie – perfusie (meestal met paraformaldehyde en glutaraaldehyde)
2. Postfixatie: immersie (osmiumtetroxide)
3. Ontwateren
4. Inbedding in hars
5. Trimmen + snijden ultradunne sneden
6. Contrasteren



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Maken van preparaten voor elektronenmicroscopie
Transmissie elektronen microscopie

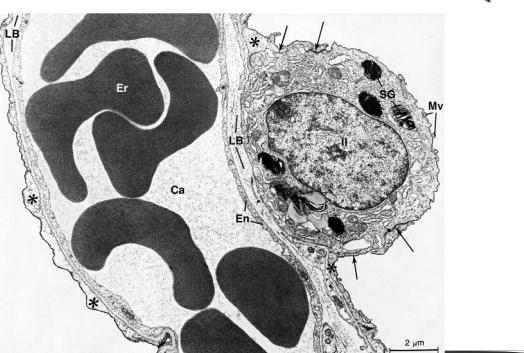
1. Fixatie: immersie – perfusie (meestal met paraformaldehyde en glutaraaldehyde)
2. Postfixatie: immersie (osmiumtetroxide)
3. Ontwateren
4. Inbedding in hars
5. Trimmen + snijden ultradunne sneden
6. Contrasteren



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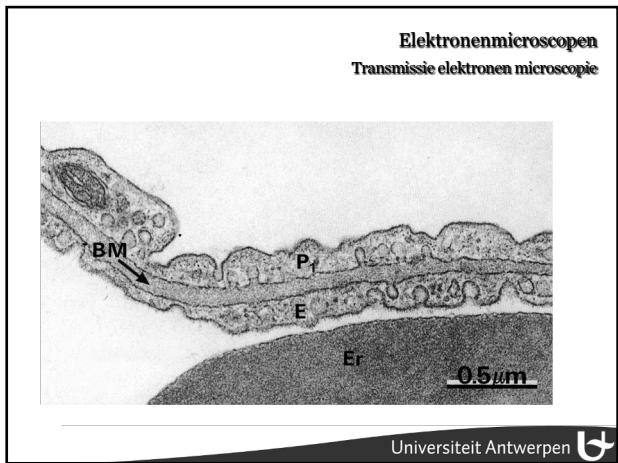
Elektronenmicroscopen
Transmissie elektronen microscopie



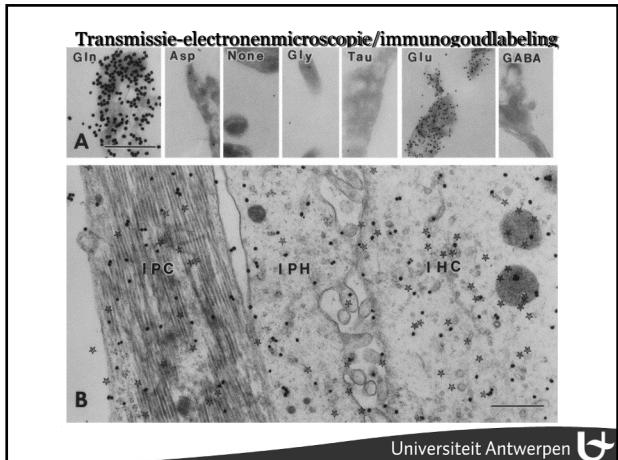
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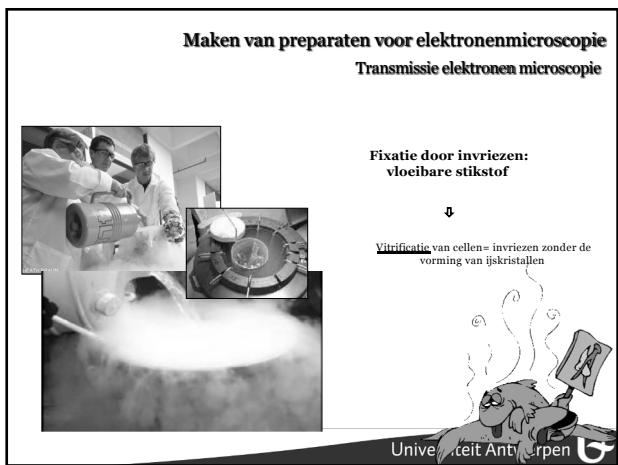
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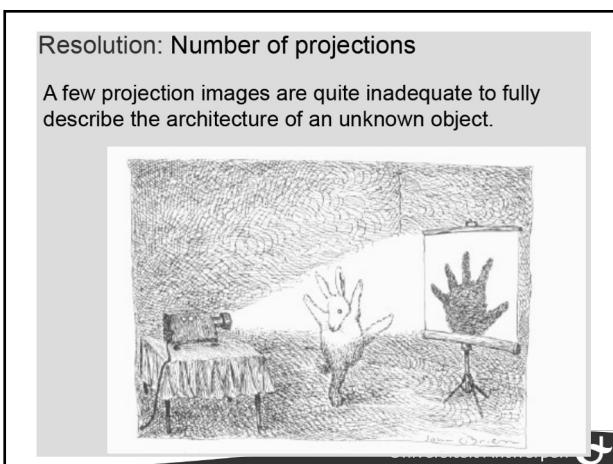
Microscopische technieken



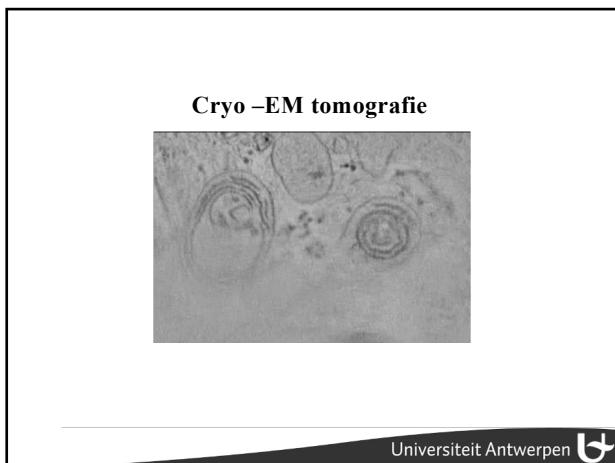
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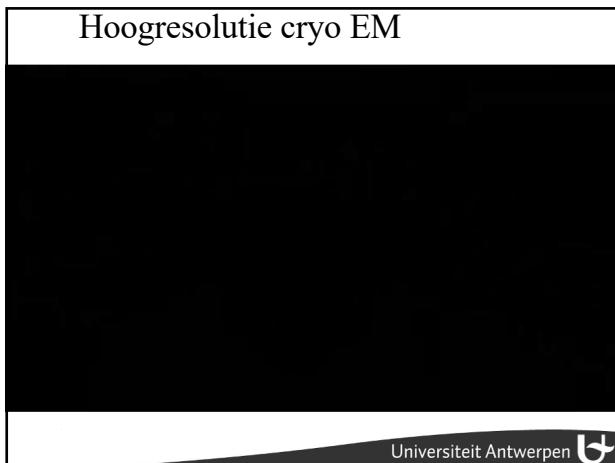
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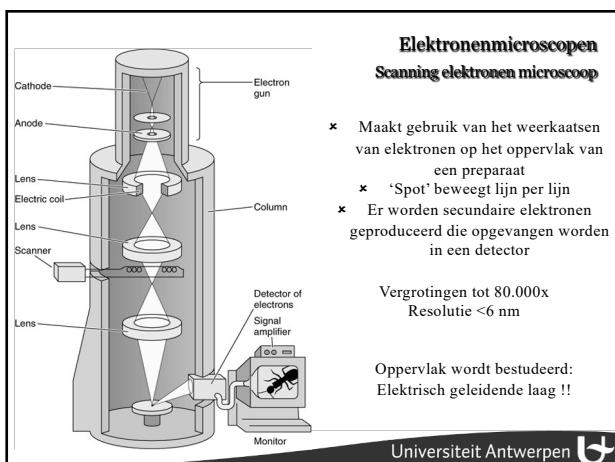
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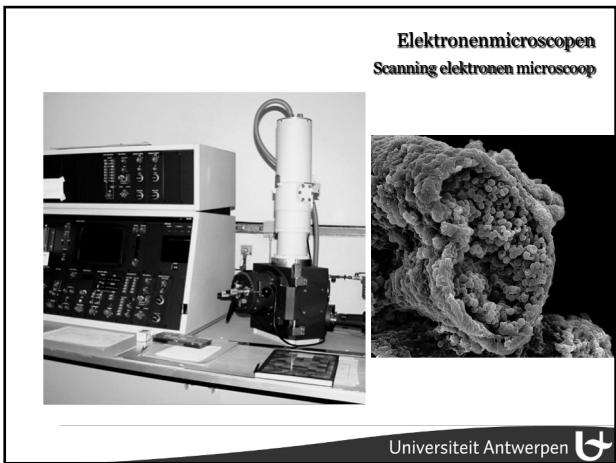
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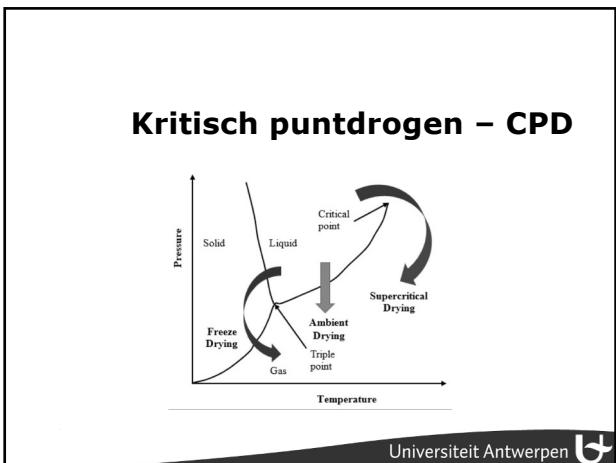
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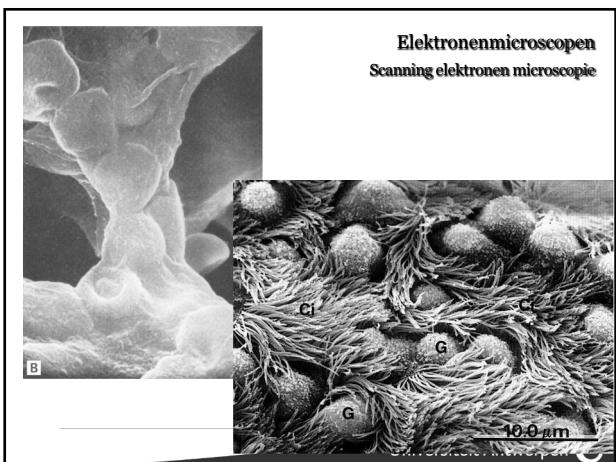
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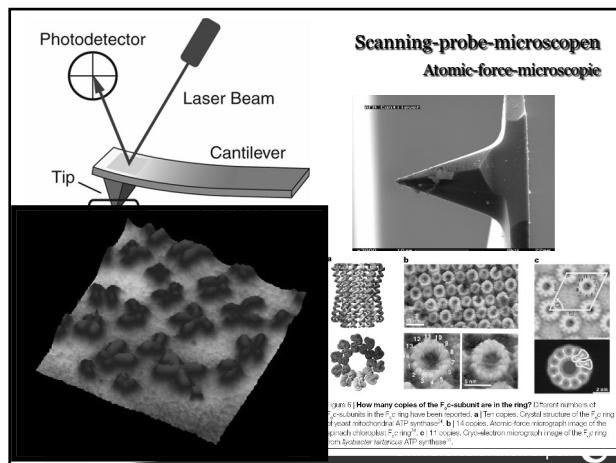


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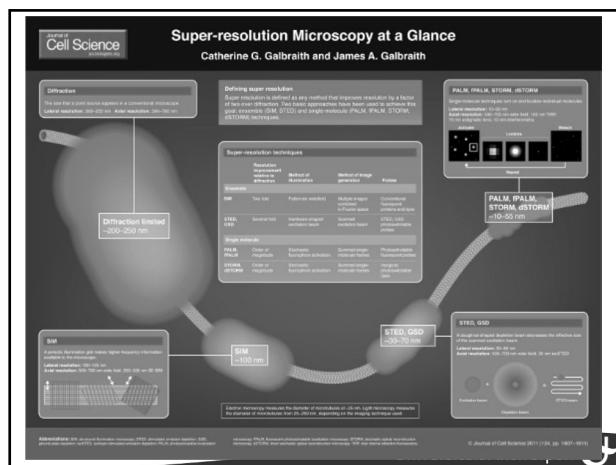


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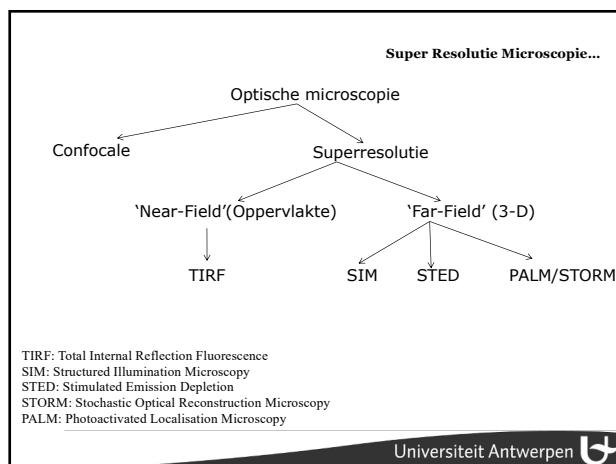
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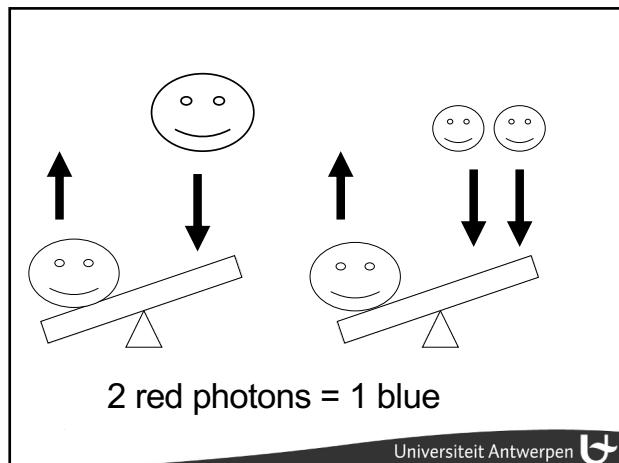
**'Single molecule lokalisatie' microscopie:
PALM en STORM**

- Meerdere cycli
- Uiteindelijke beeld is een kaart met coördinaten van de fluorochromen
- Resolutie = 30 nm

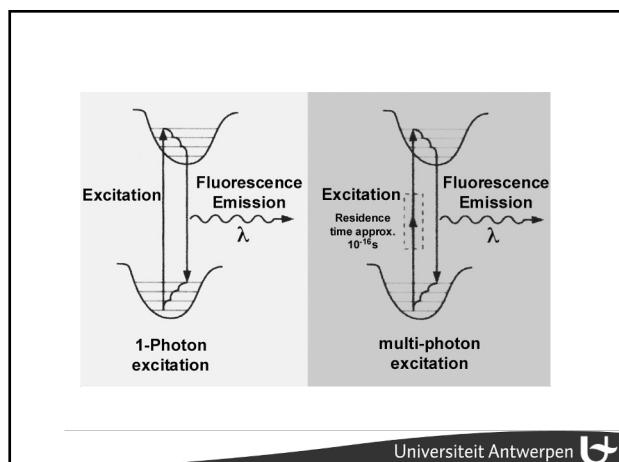
Microtubules (green) and mitochondria (magenta) in BS-C-1 cells. Showing the conventional wide-field fluorescence image, the 2D STORM image, and a 50 nm thick slice of the 3D STORM image which resolves the hollow shape of the mitochondrial outer membrane.

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Microscopische technieken

MULTI-PHOTON MICROSCOPY

Advantages of multi-photon microscopy

Confocal Two-photon

- No absorption of excitation light in out-of-focus planes
- Red-shifted light is less prone to scattering and absorption
- Absence of pinhole allows detection of scattered fluorescence light

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LIGHT SHEET MICROSCOPY

PLANE ILLUMINATION BY CYLINDRICAL LENS OR LASER SCANNING

Excitation Objective lens Fluorescence

Cylindrical lens Laser scanning Light sheet Sample Detection

SPIM (Munkan et al. [2]) DLSLM (Heller et al. [3])

Illumination Focal plane

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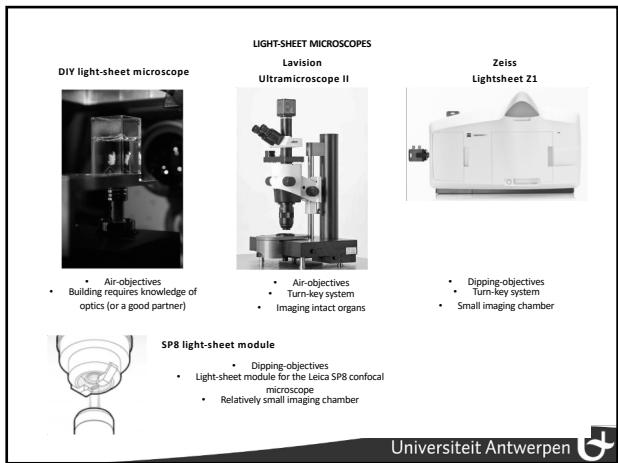
Scan CONFOCAL MICROSCOPE VS. LIGHT-SHEET MICROSCOPES

	Photobleaching	Speed
Confocal microscopy	High	Slow (exposure 3-4 s)
Light-sheet microscopy	Low	Fast (exposure 200 ms)

↓ 20X

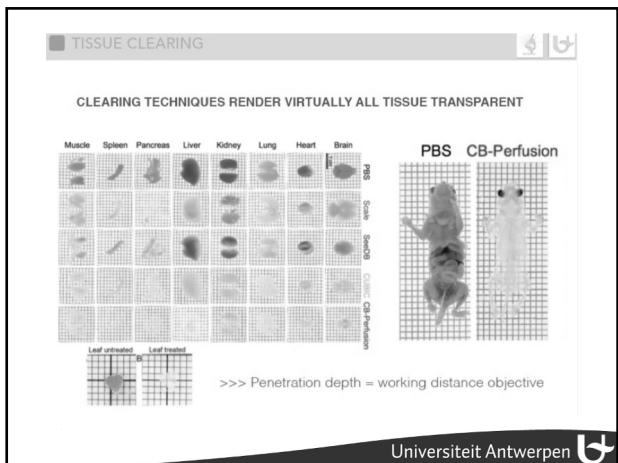
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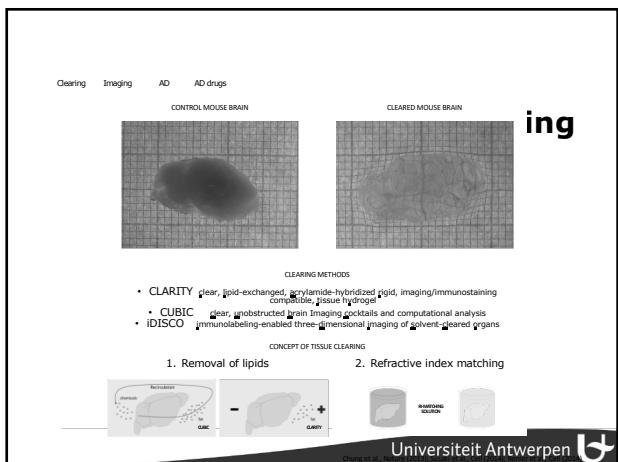
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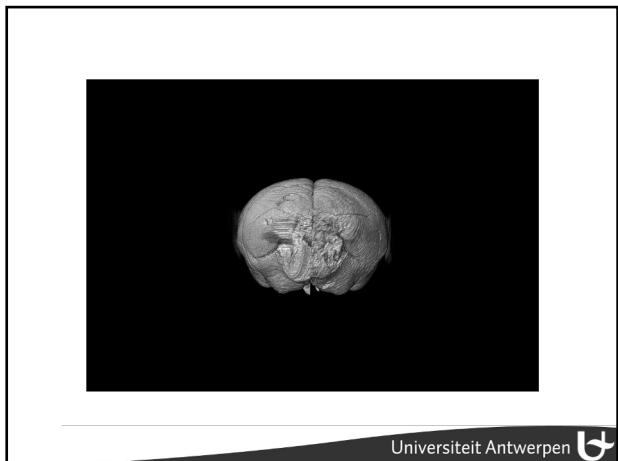
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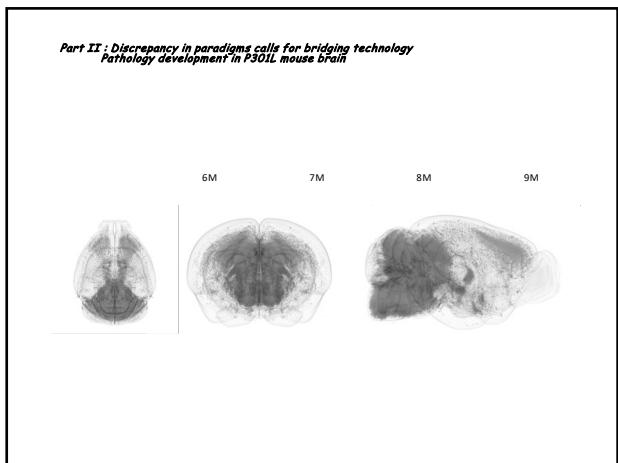
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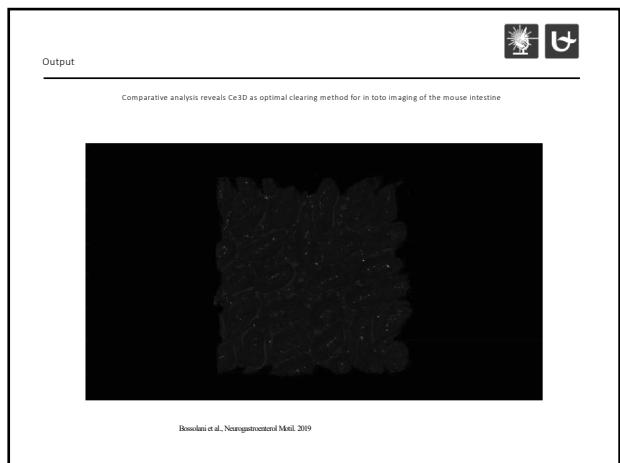


98

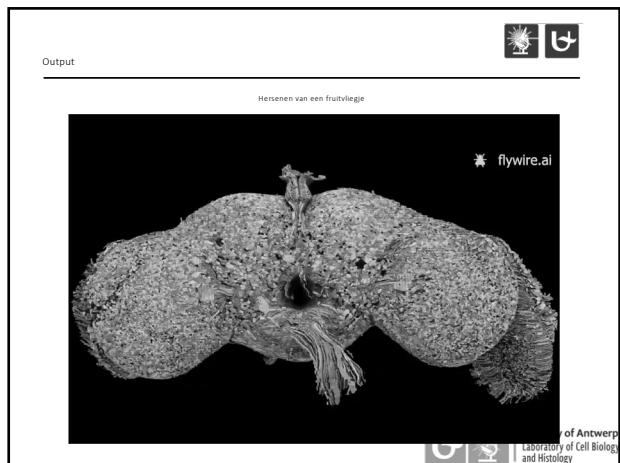


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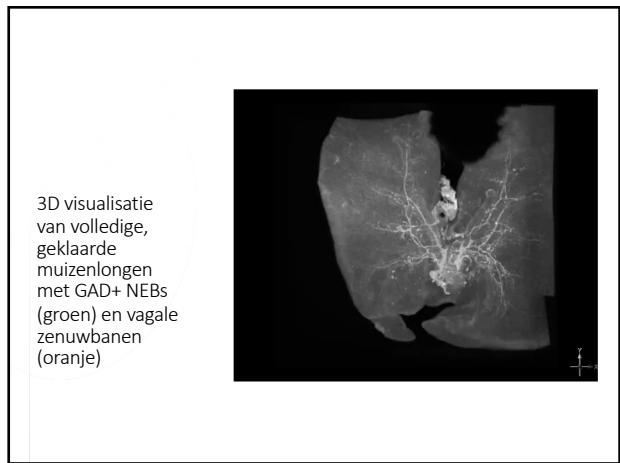
Microscopische technieken



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Voorbeeld meerkeuzevraag

1. Je wil bepalen of toediening van farmaca een invloed heeft op cellen in cultuur en wil dit fysiologisch proces volgen. Hiervoor gebruik je:

- a) Een transmissie elektronenmicroscoop
- b) Een scanning elektronenmicroscoop
- c) Een helderveld lichtmicroscoop
- d) Een confocale spinning disk microscoop

2. Je wil nagaan of 2 neurotransmitters in dezelfde vesikel aanwezig zijn in een axon. Welke techniek gebruik je?

- a) Immunokleuring en fluorescentie
- b) Immunokleuring en SEM
- c) Immunokleuring (fluorescentie) en TEM
- d) Immunokleuring (Au) en TEM



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Voorbeelden bijvraag

1. Geef me de belangrijkste verschillen in concept bij conventionele fluorescentie microscopie en confocale microscopie.
2. Waarvoor zou je DIC microscopie gebruiken.
3. Zijn de redenen om de samples te dehydrateren tijdens de voorbereiding voor SEM en TEM dezelfde? Verklaar.



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