Laboratory demonstrations

PLANT DNA FLOW CYTOMETRY

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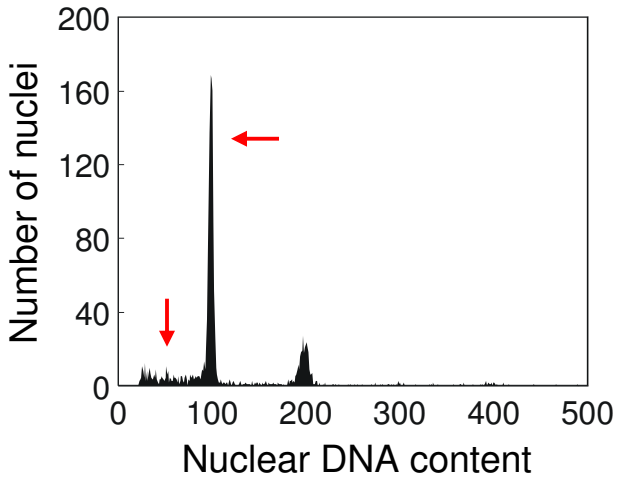
Analysis of nuclear DNA content

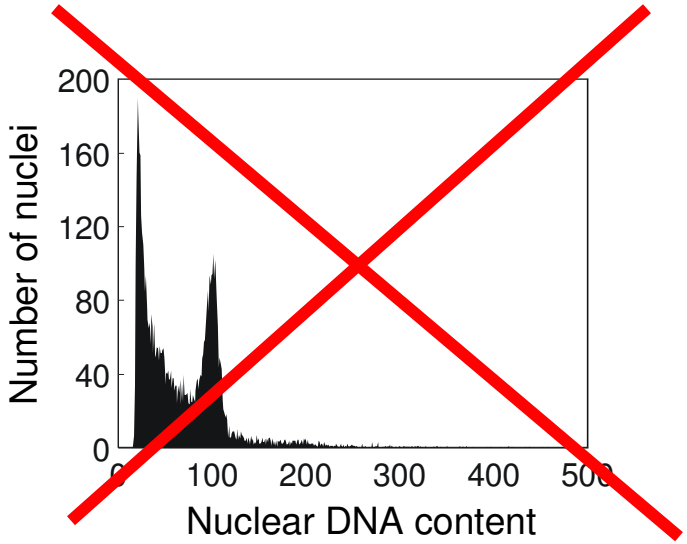
- **Relative nuclear DNA content**
  - Ploidy (*x*)
  - Aneuploidy
  - Mixoploidy
  - Endopolyploidy
  - Cell cycle kinetics
- **DNA content in absolute units**
  - Nuclear genome size (pg DNA, bp)
- **AT/GC ratio**

What do we need for reliable analysis?

- **„Nice“ histograms of nuclear DNA content!**
  - •Narrow DNA peaks (low coefficient of variation, CV)
  - •Negligible debris background

Golden rule: Choose **fresh**, **young** and **healthy** leaf tissue
(but be prepared for „difficult“ species!)





Choosing a proper method …

- **Choice of the method involves selection of**
  - Nuclei isolation buffer
  - Procedure for release of intact nuclei from cells
  - Fluorochrome to stain nuclear DNA
  - Method of standardization

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Secrets of nuclei isolation buffers

- **Main functions**
  - Release of nuclei from cells
  - Preservation of integrity of isolated nuclei
  - Inhibition of nuclease activity

- **Typical components**
  - Chromatin stabilizers (polyamines, divalent cations Mg^{2+}, Ca^{2+})
  - Sucrose, mannitol
  - Metal chelators (EDTA, EGTA) – not in Mg^{2+} or Ca^{2+} buffers!
  - Salts (KCl, NaCl)
  - Reducing agents (mercaptoethanol, DTT, PVP)
  - Detergents (Triton X-100, Tween 20)
  - Buffers (TRIS, HEPES)

How to choose the best nuclei isolation buffer?

- **By trial and error!**
  - There is no simple rule to tell which buffer will work best
- **Consider the following**
  - DNA fluorochrome that will be used to stain nuclear DNA
  - Need to suppress the action of secondary metabolites
  - Need to avoid precipitation of mucous substances

„Top six" of nuclei isolation buffers

- **Galbraith buffer (Galbraith *et al*. 1983)**
  - $MgCl_2$ / sodium citrate buffer; usually satisfactory results
- **Bergounioux *et al*. (1986)**
  - „Tissue culture salts"; works well with most of materials
- **LB01 (Doležel *et al*. 1989)**
  - Polyamine buffer; works well with most of materials
- **Otto buffers (Otto 1991) - two-step procedure!**
  - Citric acid; excellent resolution with most of materials
- **Arumuganathan and Earle (1991)**
  - $MgSO_4$ buffer; procedure requires centrifugation
- **TRIS-$MgCl_2$ (Pfosser *et al*. 1995)**
  - Simple composition; works well with some materials

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Materials used for analysis of nuclear DNA content

- **Type of material**
  - Fresh materials
  - Fixed materials (formaldehyde or ethanol : acetic acid fixatives) *(not suitable for genome size or AT/GC content!)*
  - Special materials *(dry seeds, herbarium vouchers)*

- **Quantity**
  - Depends on the method (20 – 100 mg / sample) *(more is not always better!)*

How to release intact nuclei from plant cells?

- **Fresh materials**
  - Mechanical homogenisation using sharp razor blade or scalpel *(not always suitable for suspension-cultured cells)*
  - Lysis of protoplasts in a hypotonic buffer *(laborious but excellent histograms)*

- **Fixed materials**
  - Formaldehyde ⇨ mechanical homogenisation
  - Ethanol : acetic acid (3:1) ⇨ enzymatic hydrolysis of cell walls

- **Special types of materials**
  - Dry seeds ⇨ sand paper
  - Herbarium vouchers ⇨ mechanical homogenisation

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Fluorescent stains for DNA

Fluorescent Dye	Primary Binding Mode	Wavelength (nm)*	
		Excitation	Emission
Ethidium bromide**	Intercalation	530	605
Propidium iodide**	Intercalation	540	615
Hoechst 33258	AT-binding	365	465
Hoechst 33342	AT-binding	360	460
DAPI	AT-binding	365	450
DIPI	AT-binding	365	450
Chromomycin A3	GC-binding	445	570
Mihtramycin	GC-binding	445	575
Olivomycin	GC-binding	440	560

*Dye-DNA complex

**Binds also to double stranded RNA!

How to stain nuclear DNA?

- **Consider the following**
  - Type of DNA fluorochrome
    - Genome size ⇨ only DNA intercalator (!)
    - Ploidy, cell cycle ⇨ any DNA fluorochrome
    - AT/GC ratio ⇨ two separate measurements (DNA intercalator, AT- or GC-binding dye)
  - Optimal concentration
  - When to add the fluorochrome
    - Fluorochrome present in the nuclei isolation buffer
    - Fluorochrome added to nuclei suspension

Choosing a proper method …

- **Choice of the method involves selection of**
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  - Procedure for release of intact nuclei from cells
  - Fluorochrome to stain nuclear DNA
  - **Method of standardization**

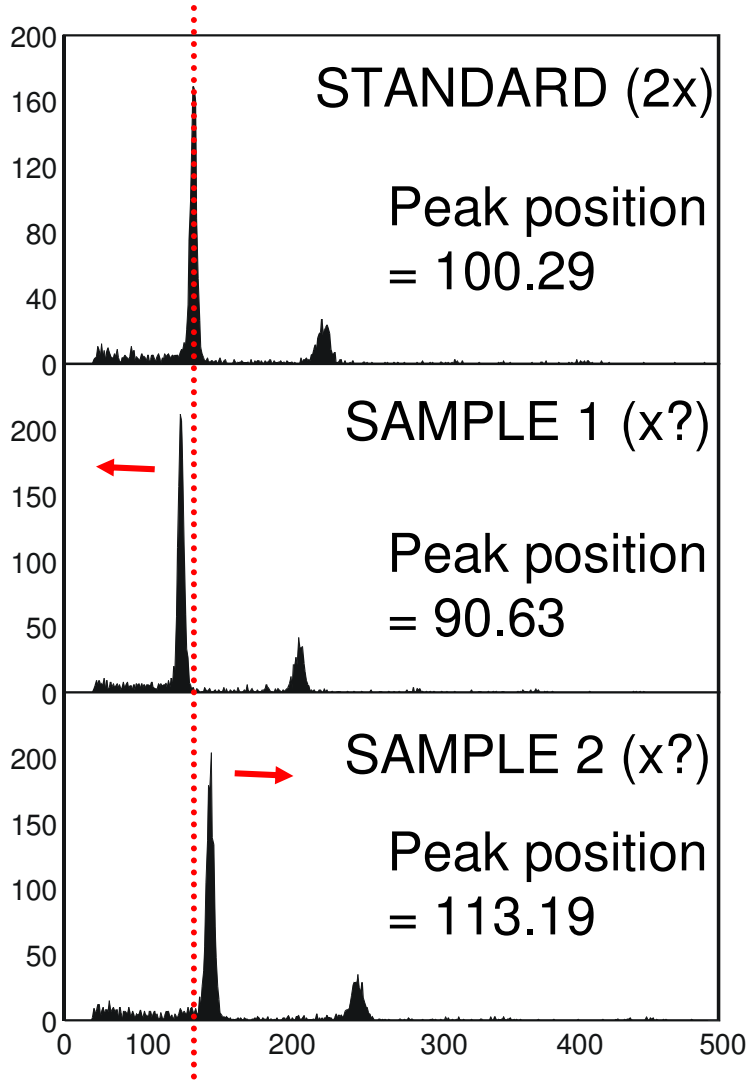
The use of standards recommended!

- **Flow cytometry estimates DNA content in relative units (channel numbers)**

- **DNA peak position may vary due to**
  - Random instrument drift
  - Variation in sample preparation and staining procedure

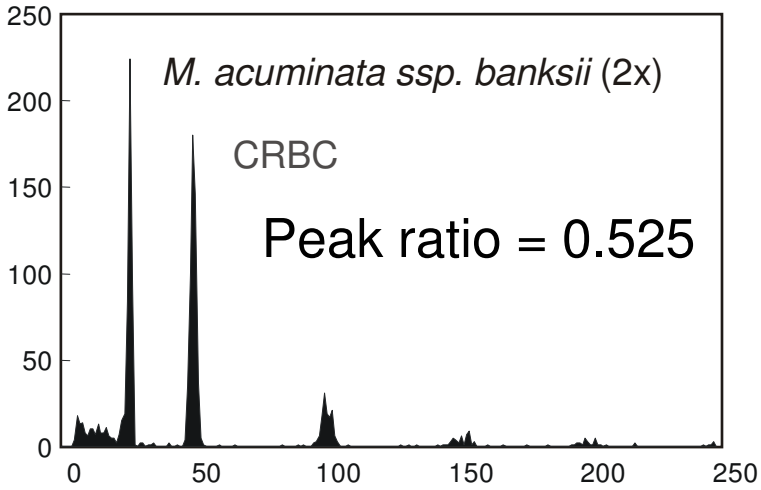
- **Standardization**
  - External
    - Rapid screening of ploidy levels
  - Internal
    - Precise estimation of DNA ploidy levels, incl. aneuplooidy
    - Determination of genome size

External standard ?



- **YES – if acceptable**
  - Rapid and convenient, but:
  - Precise ploidy determination not possible
  - Larger fluctuation of peak position with non intercalating dyes

Internal standard?



Variation in DNA peak ratio

n	13
Mean	0.527
S.D.	0.11

- **YES – if possible *(and needed)***
  - Permits precise estimation of DNA ploidy level
  - Other species may serve as standard
  - More laborious:
    - Two samples in one
    - Requires calculation of peak ratio

Trying to make a good sample …

- **The rule of seven**
  - Test different tissues (if possible); in case of non-fixed materials the tissues must be healthy and fresh !!!
  - Try different ratios of material : isolation buffer
  - Compare different nuclei isolation buffers
  - Test different detergents at different concentrations
  - Check the effect of reducing agents
  - Prolong nuclei incubation in the isolation buffer
  - Pellet the isolated nuclei and resuspend them in a fresh buffer

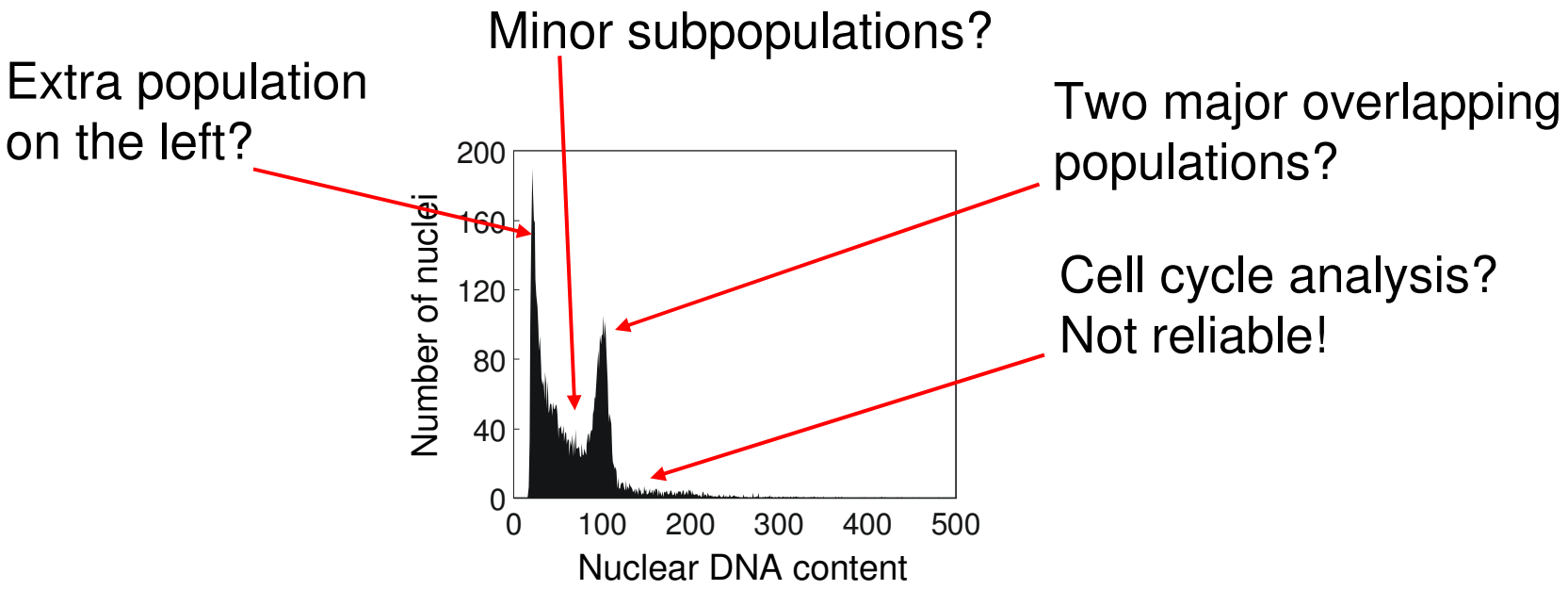
DNA flow cytometry is not as easy as it seems!

- **Relative simplicity of DNA flow cytometry may be deceiving and lead to erroneous conclusions**

- **Typical problems**
  - Excessive debris background, high CV of DNA peaks
  - Problems with standardization
  - Presence of non-cycling and endopolyploid cells
  - Contamination of material with other organisms

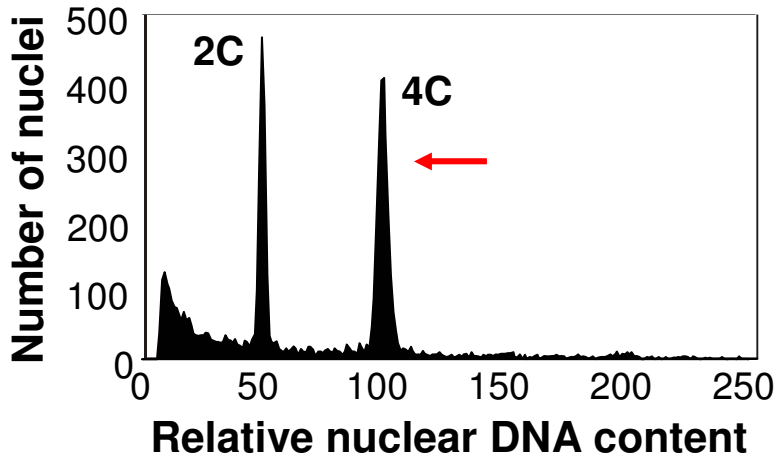
It is a hard life with a bad sample …

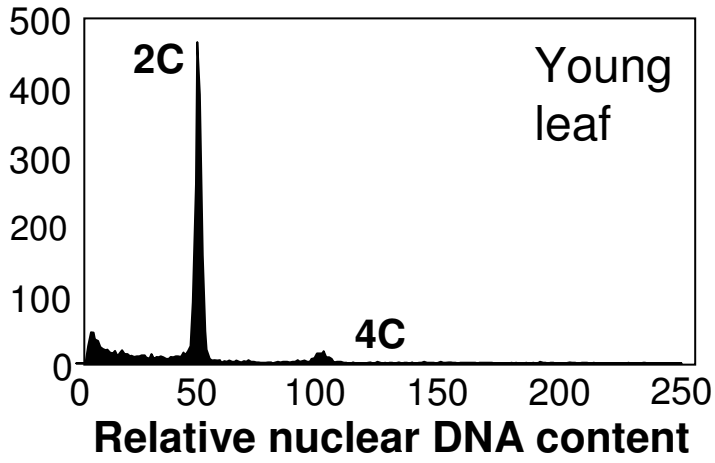
- **Interpretation of DNA content histograms**
  - Narrow DNA peaks and negligible debris background ⇨ EASY
  - Broad DNA peaks and excessive debris background ⇨ DIFFICULT / IMPOSSIBLE



Diploid or mixoploid?

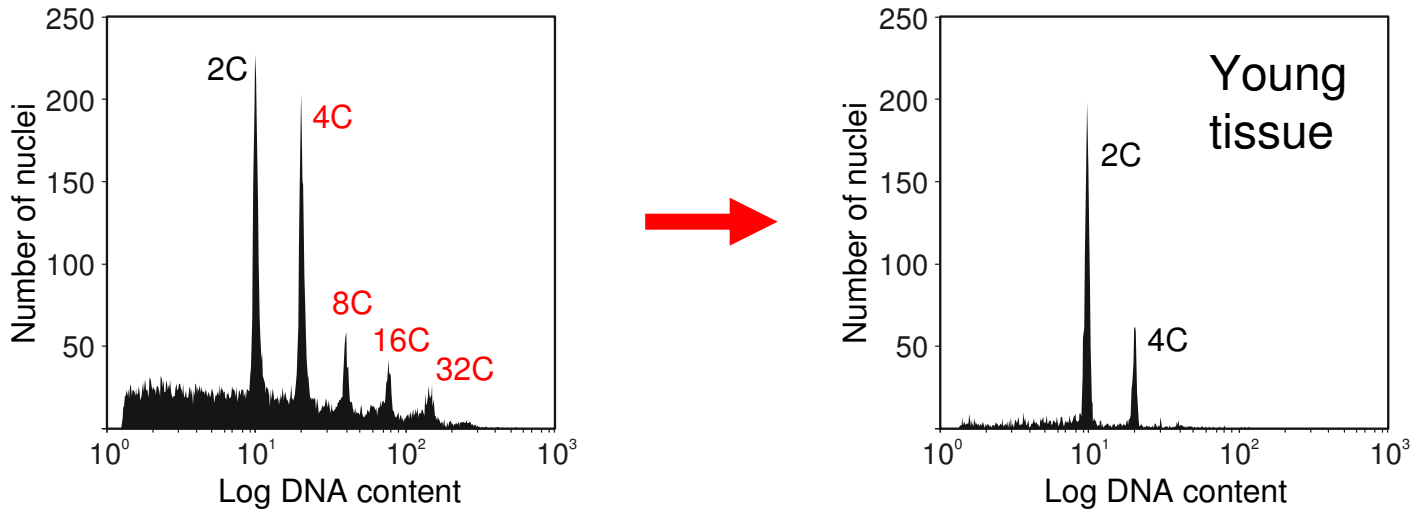
- **Not always easy to know!**
  - Cells with 4C DNA content may be 2*x* cells in G_2 or 4*x* cells in G_1
  - Useful hint: use young, actively growing tissues to exclude non-cycling cells in G_2



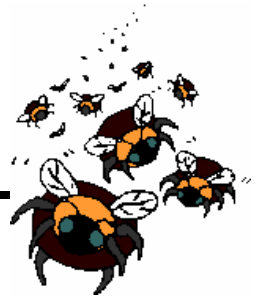


Mixoploid or endopolyploid ?

- **Not always easy to know!**
  - Polyploid and endopolyploid cells may have the same DNA content
  - Useful hint: use young, non-differentiated, actively growing tissues to exclude endopolyploidy



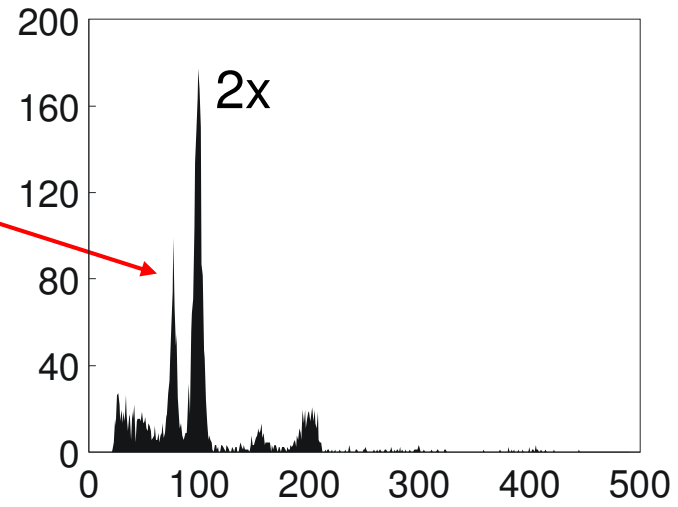
Beware of insect parasites!



- **Nuclei of a parasite may be isolated together with the nuclei of your material**

- **Check carefully any result with unexpected peaks!**
  - Clean the leaf and make a new sample *(not always successful)*
  - Use another leaf or plant *(preferable)*

Aneuploid subpopulation?
No! Insect parasite.



GOOD LUCK!