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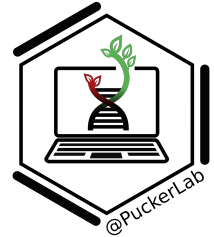

Prof. Dr. Boas Pucker

Long Read Genomics

- Plant Genome Sequencing with ONT

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Availability of slides

- All materials are freely available (CC BY) - after the lectures:
  - GitHub: https://github.com/bpucker/LRG

- Questions: Feel free to ask at any time

- Feedback, comments, or questions: pucker[a]uni-bonn.de

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Repetition

- Sequencing technologies:
  - Sanger
  - Illumina
  - PacBio
  - ONT
- Trends in plant genomics
- Cloud computing

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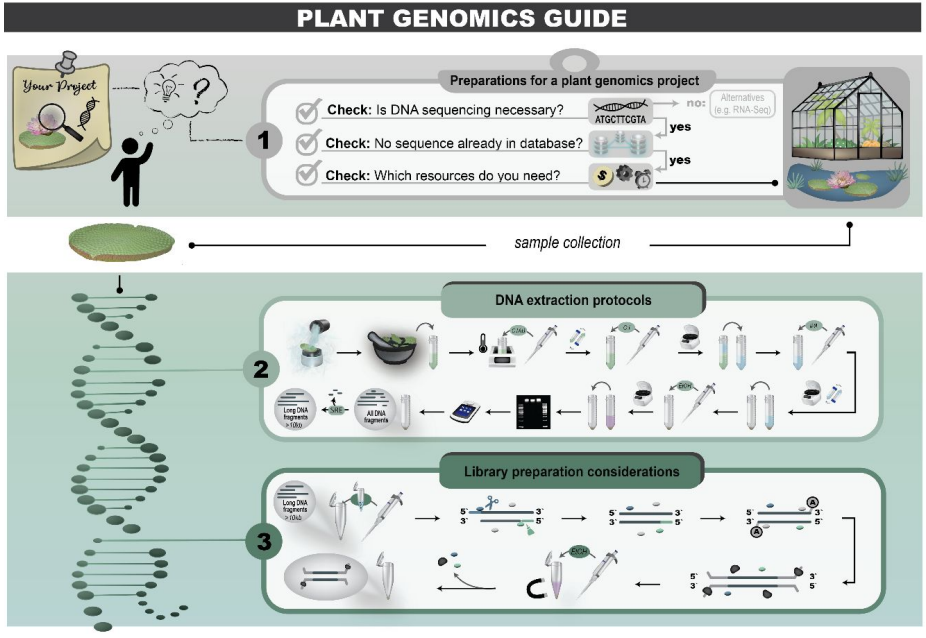
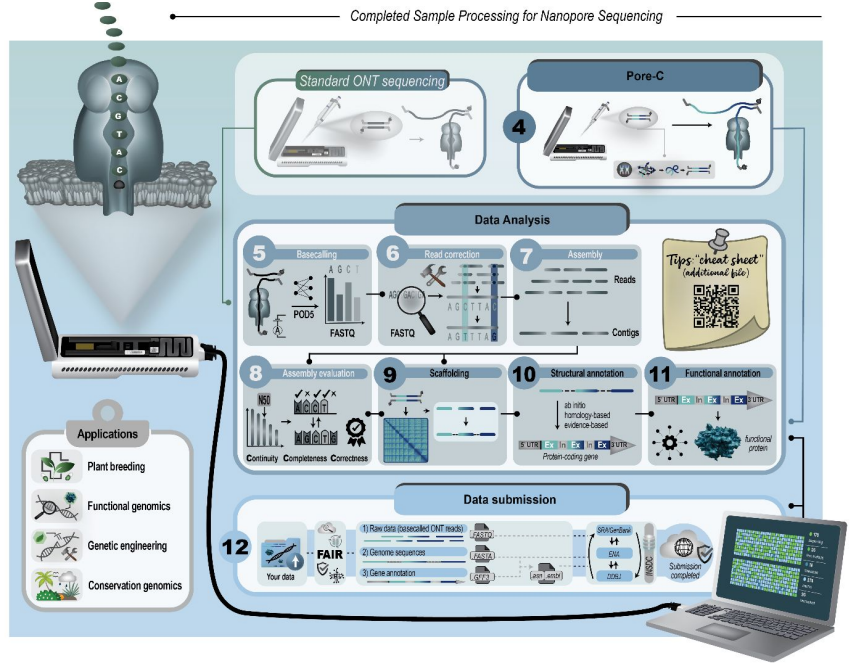
Plant Genomics Workflow





Supplementary file with all commands!

de Oliveira et al., 2025: 10.20944/preprints202508.1176.v2

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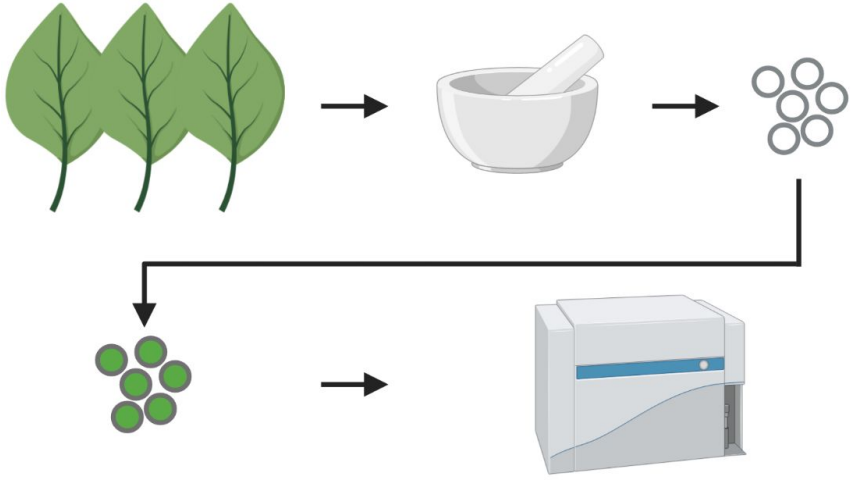
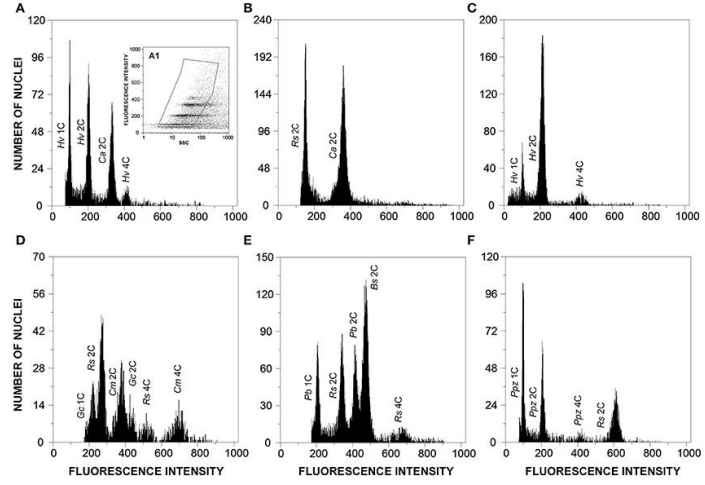
Considerations & preparations

- What is the expected/estimated genome size?
- What is the ploidy of the species?
- Which individual should be sequenced?
- Which plant parts are suitable for DNA extraction?
- What materials are needed for DNA extraction and sequencing?

Genome size - 1

- Genome sequences of closely related species
- Flow cytometry is used to measure genome size biochemically
  - C-value
- Databases for plant genome sizes: https://cvalues.science.kew.org/



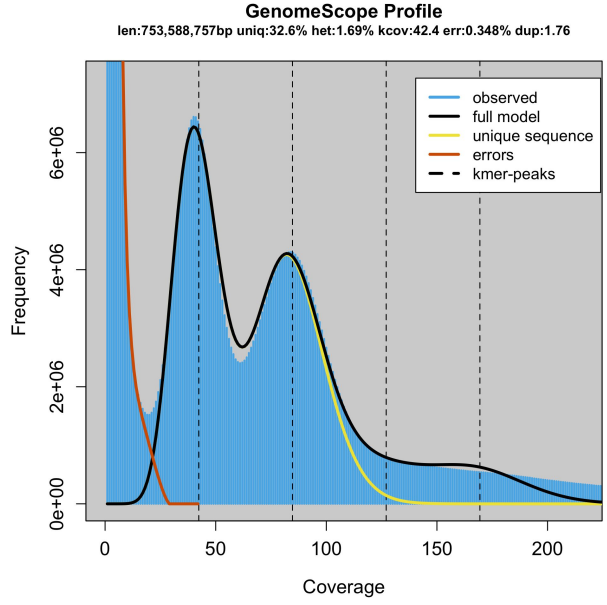


https://doi.org/10.3389/fpls.2014.00422

Genome size - 2

- Tools for genome size estimation based on short reads
  - K-mer-based: GenomeScope2, findGSE, gce

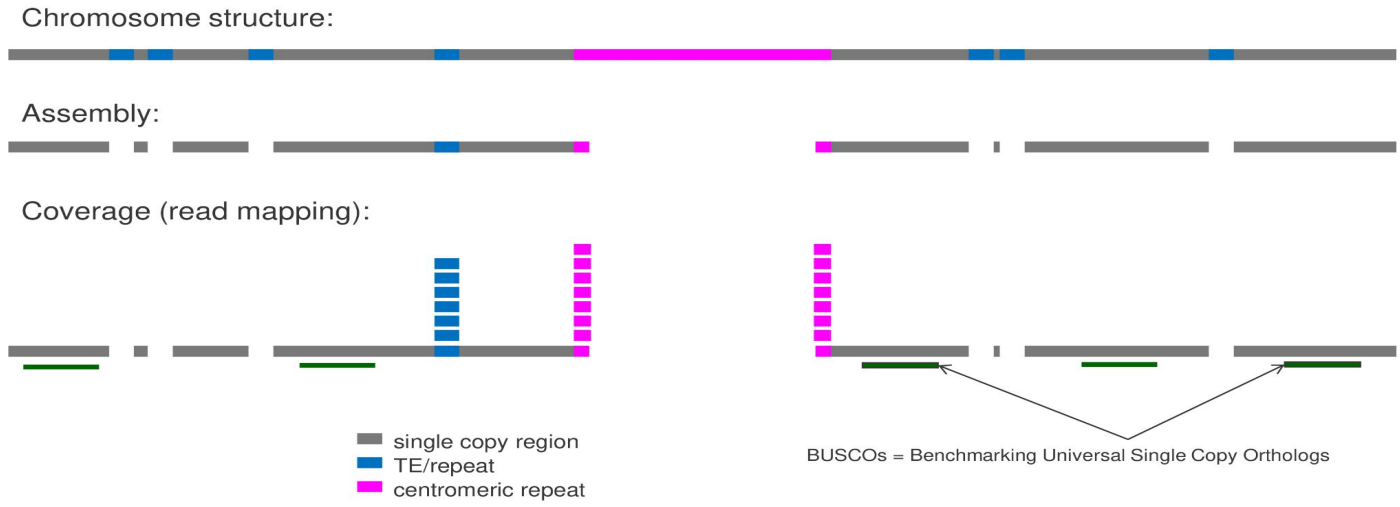
```
ACGAAGCCATAT
ACGAAGC
 CGAAGCC
  GAAGCCA
   AAGCCAT
    AGCCATAT
```



https://github.com/schatzlab/genomescope

Genome size - 3

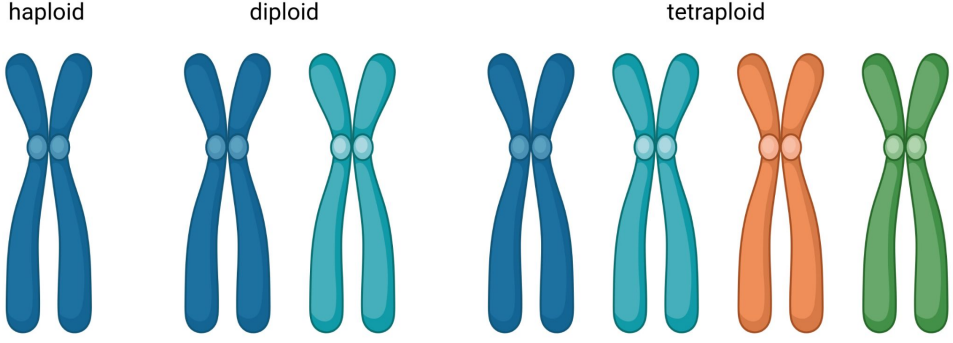
- Tools for genome size estimation based on short/long reads
  - Mapping-based: MGSE, Gnodes

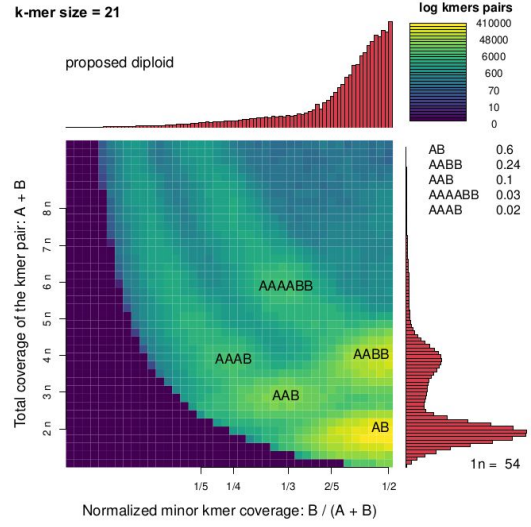


Natarajan et al., 2024: 10.1186/s12864-025-11640-8

Ploidy - 1

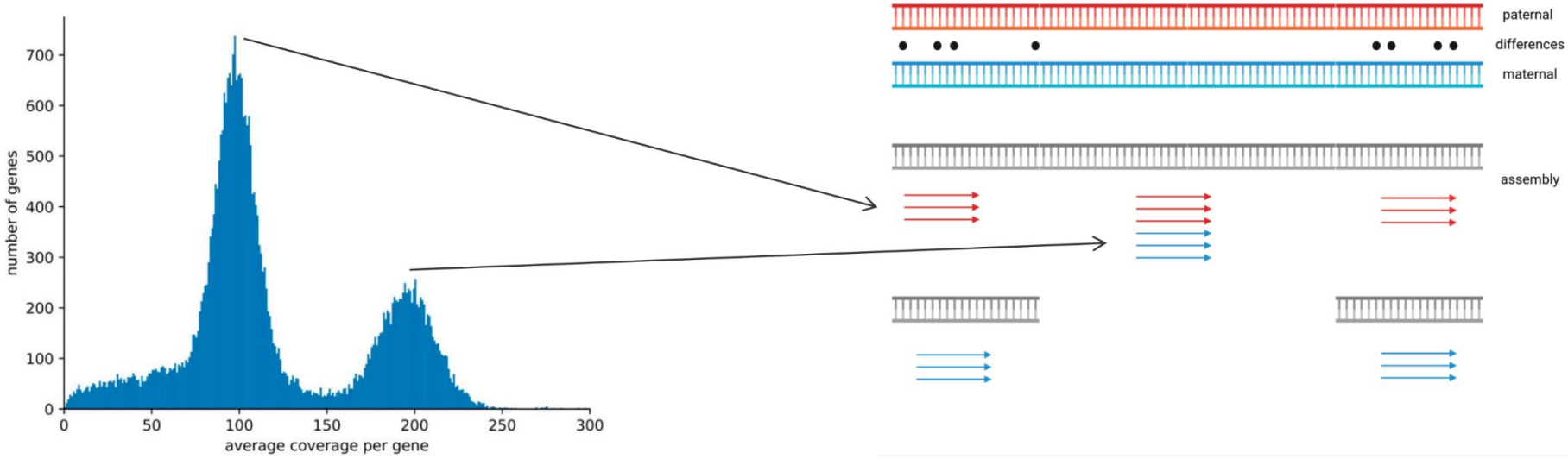
- Ploidy = copy number of same chromosome
- Many plants are polyploid
- Some polyploid plants have close diploid relatives
- Smudgeplots to analyze polyploidy based on short reads





Siadjeu & Pucker et al., 2020: 10.3390/genes11030274

Ploidy - 2



Siadjeu & Pucker et al., 2020: 10.3390/genes11030274

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Picking an individual

- Plant should survive the sampling for DNA extraction
- Plant should be a good representation of the species
- Source of the plant is important:
  - Restrictions through material transfer agreements (MTAs)?
  - Restrictions through Nagoya protocol / Access and Benefit Sharing (ABS) law

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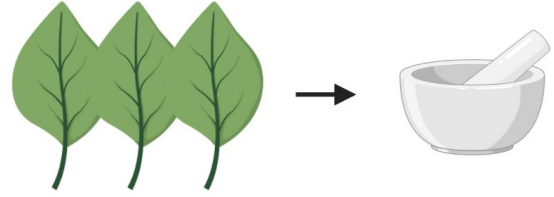
Plant part for DNA extraction

- Young leaf is often a good choice
- Small cells result in higher density of nuclei per weight
- Concentration of specialized metabolites should be low
- Amount of sugar should be low
- Amount of chloroplast should be low
- Sample should not be contaminated with bacteria/fungi



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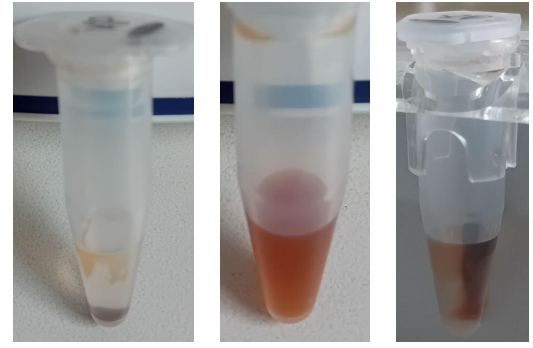
Material for DNA extraction

- Cetyltrimethylammonium-bromide (CTAB) buffer
- beta-mercaptoehtanol (ß-ME)
- Dichlormethane (replaces chloroform)
- TrisEDTA (TE) + RNase A
- OPTIONAL: Short Read Eliminator (SRE) kit

Material required for sequencing

- DNA repair (companion module)
- Magnetic beads for purification
- Library preparation kit
- Sequencing kit
- Wash kit with DNase



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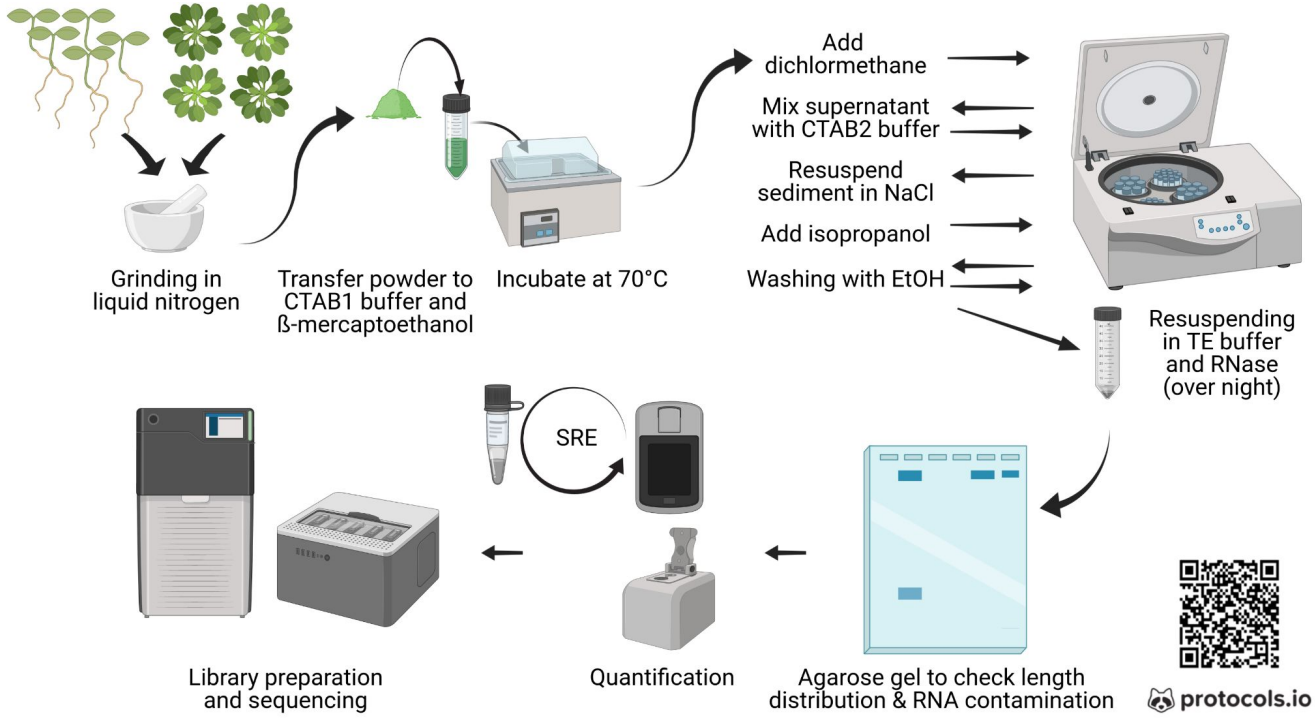
Workflow

		task	consumed time	hands-on time	equipment	estimated costs of consumables	estimated costs of lab equipment
A		plant incubation in darkness	2-3d	1h			
B		non-destructive sampling	-	1h			
C		DNA extraction	1d	8h	waterbath, centrifuge	$50	$1000 $8000
D		quality control	1h	1h	NanoDrop, Qubit	$20	
E		short fragment depletion	2h	1h	centrifuge	$50	
F		quality control	1h	1h	NanoDrop, Qubit	$20	$5000 $5000
G		library preparation & sequencing	1-5d	4-16h	centrifuge, magnetic rack, sequencer	$3000	$250 $1000
H		basecalling	1d	1h	computer with GPU		$3000
I		assembly	1-15d	1h			
J		polishing	1-5d	1h	compute cluster / cloud		
K		annotation	1-5d	1h			
L		data submission	2h	2h	fast internet connection		

Pucker et al., 2022: 10.1017/qpb.2021.18

DNA extraction workflow



ONT sequencing devices

MinION

PromethION 2 Solo

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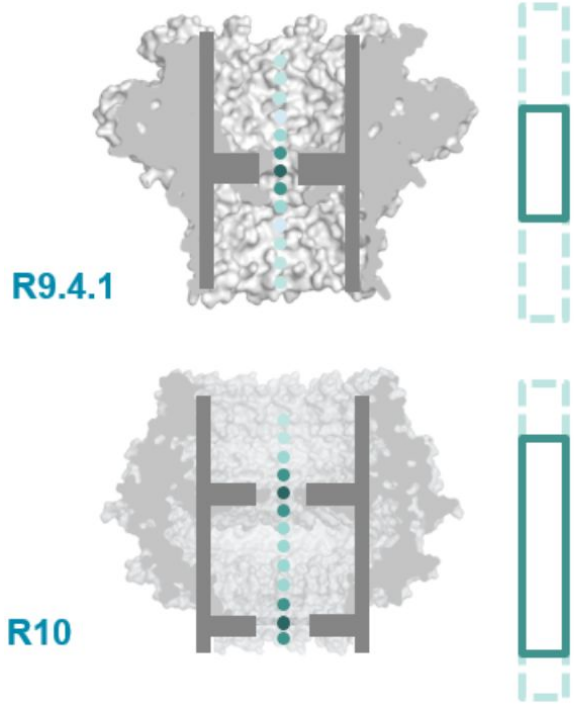
Flow cells: MinION vs. PromethION

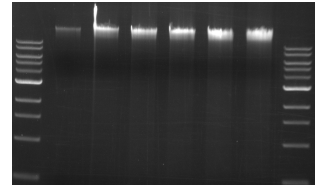
Nanopore comparison



https://nanoporetech.com/about-us/news/r103-newest-nanopore-high-accuracy-nanopore-sequencing-now-available-store

Quality control

- Agarose gel electrophoresis

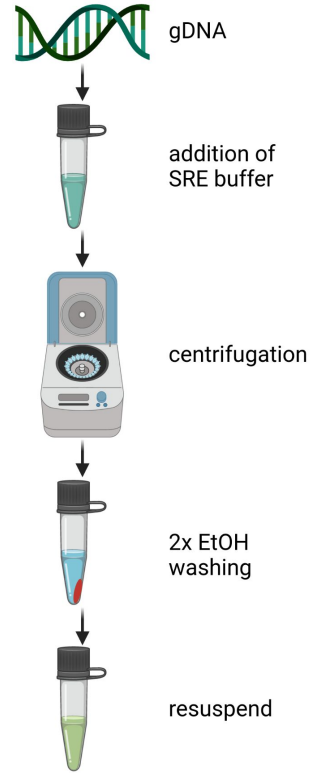


- Photometric measurement via NanoDrop

- Quantification with Qubit

Short Read Eliminator (SRE)

- Proprietary salt mix for DNA precipitation
- Removal of <10kb DNA fragments
- Depletion of <20kb DNA fragments
- ONT read length distribution can be substantially improved



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DNA repair

- Repairing single strand DNA breaks

- Repairing DNA ends (3’-A overhang required for adapter ligation)

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Library preparations

- Repaired DNA is subjected to library preparation
- Addition of adapters to DNA fragments (expensive ligase)
- Concentration of DNA can be quantified via Qubit measurement (optional)
  - Control step to ensure that library construction is working
- Purification of DNA with magnetic beads

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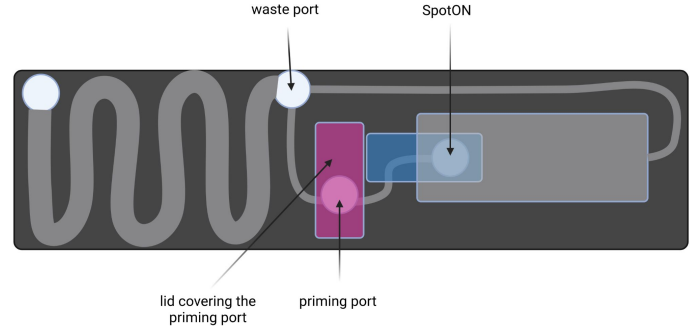
Flow cell check

- Flow cells are delivered with storage buffer (green)
- Buffer allows technical check of flow cell (number of active pores is determined)
- Number of nanopores must be >800 (MinION) or >5000 (PromethION)
- Replacement flow cells are provided if number of pores is lower

Loading flow cell

- Removal of storage buffer
- Priming of flow cell
- Introduction of air bubbles must be avoided!!!
- Fully open ports are crucial to inject solutions (avoid force)
- Video tutorial: https://www.youtube.com/watch?v=Pt-iaemrM88



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Starting sequencing

- Define the flow cell type (R10.4.1)

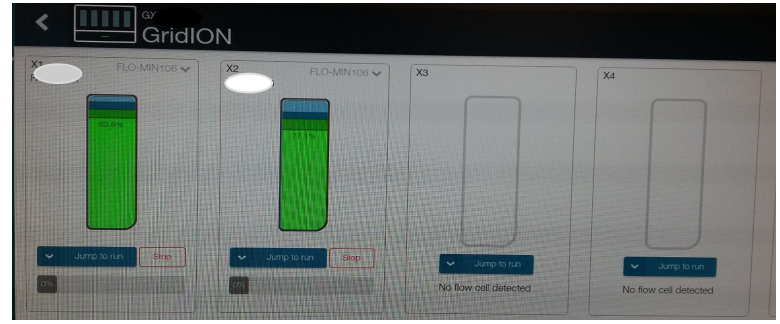
- Select parameters:
  - Make sure to keep POD5 files (default = off)
  - Define run time (default = 72h)

- Set the output location, run name, sample ID, …

- Start the sequencing some minutes after loading the flow cell
  - This allows the DNA to get into contact with the nanopores

Monitor sequencing

- Number of active nanopores can be monitored in real time
- Output is estimated in real time
- Read length distribution can be assessed
- Speed of the sequencing can be monitored
- Quality of the reads is displayed



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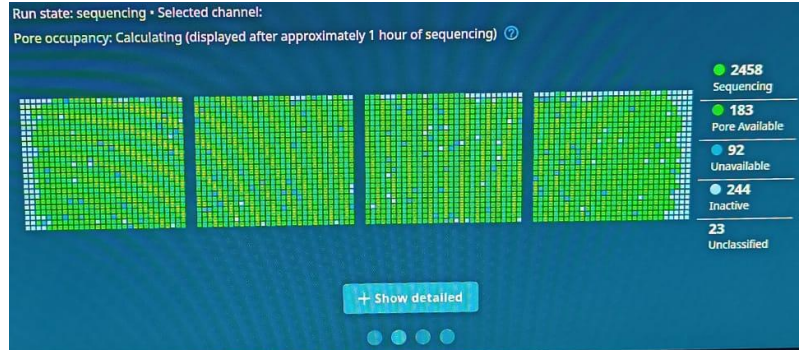
Nanopore activity

- Status of nanopores on flow cell
- Light green is good (sequencing)
- Number of sequencing nanopores change over time
- Air bubbles can destroy nanopores



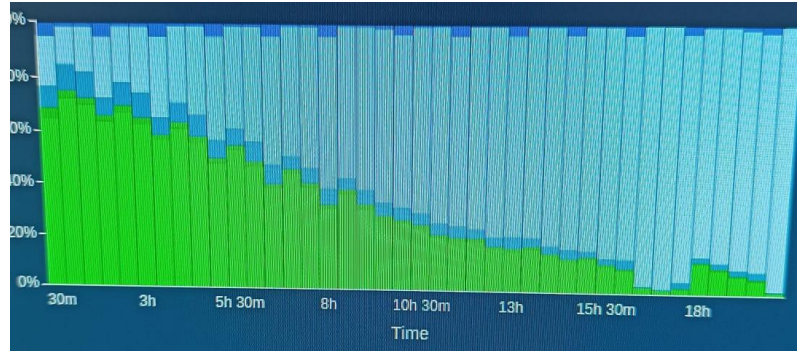
Read length distribution

- Read length distribution

- N50 summarizes length distribution

Flow cell performance over time

- Flow cell performance decreases over time
- Nanopores get blocked by DNA
- Nanopores break down due to air bubbles



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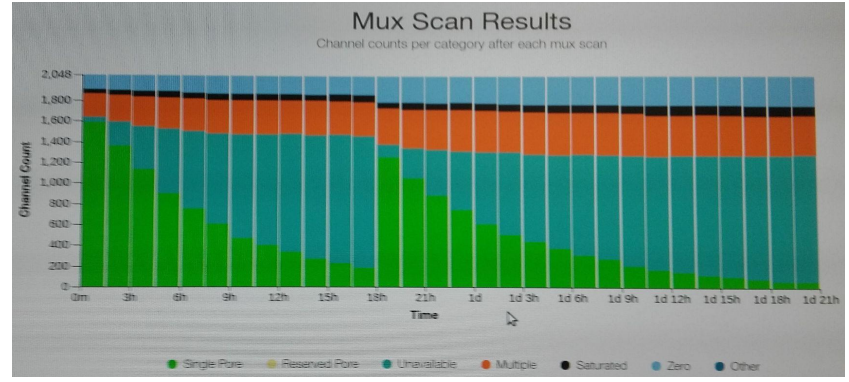
Stop & wash

- Sequencing is stopped once the number of active pores gets low
- Washing with DNase to free blocked nanopores
- Flow cells are regenerated and can be re-used
- Process can be repeated multiple times (3-5x)



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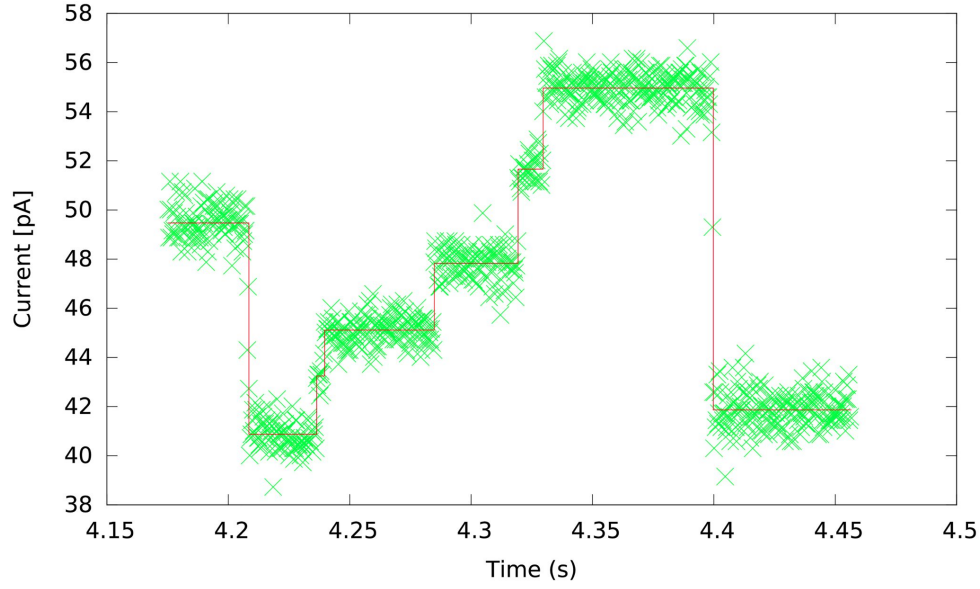
Basecalling

- Electric signal is converted into sequence information (bascalling)
- Algorithmic improvement lead to higher read accuracy
- Raw sequencing data need to be stored



Boza et al., 2017: 10.1371/journal.pone.0178751

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Basecalling with dorado

- Dorado allows conversion of electric signal into sequences
- Different models are available (HAC, SUP)
- Basecalling on CPUs is basically impossible
- Time estimates on GPUs: hours to days depending on dataset size

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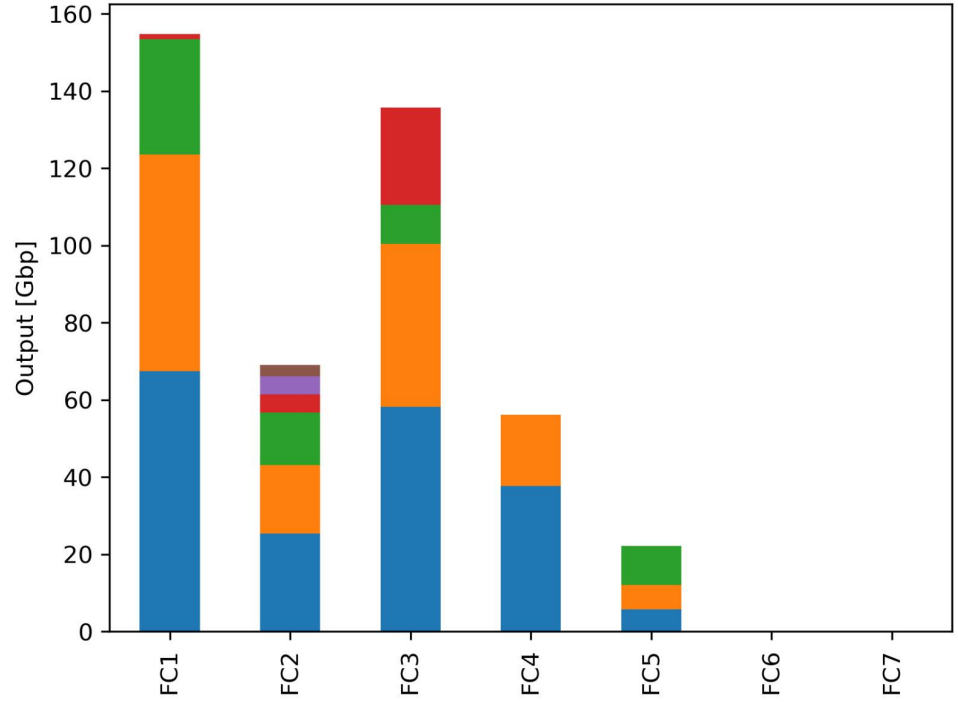
Calculating statistics

- Huge variation in output per flow cell
- Best runs: 150 Gbp
- Worst runs: 20 Gbp
- N50 for plants usually 25-65kb



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Bioinformatics / Data analysis (overview)

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Transferring files

- Filezilla: graphical user interface for file transfer protocols
  - https://filezilla-project.org/
- Scp (secure copy): command line file transfer method
- Wget: command line file transfer method
  - https://ftp.gnu.org/old-gnu/Manuals/wget-1.8.1/html_mono/wget.html
- Rsync: sophisticated file transfer method that avoids redundant transfers
  - https://wiki.ubuntuusers.de/rsync/

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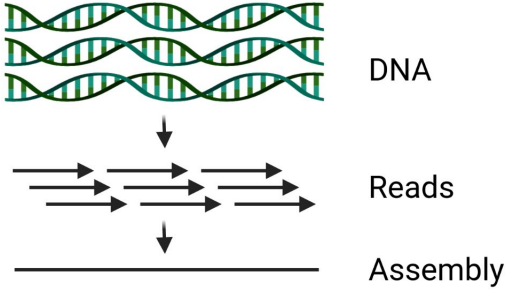
de.NBI cloud

- Virtual machine (VM) for data analysis
- Accounts are required for access (ORCID for login)
- Addition to project required for access
- User create pair of private and public keys for authentication

Genome sequence assembly

- Combination of overlapping reads to longer sequences
- Assembled sequences represent chromosomes
- Sequences are stored in FASTA file




```
>TRINITY_DN100016_c0_g1_i1
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>TRINITY_DN100019_c0_g1_i1
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>TRINITY_DN10001_c0_g1_i2
MAKVGNPIVDIETDGSVNEPESSEKNIEVSSSSTQAPESTNTTELLVNEKKAFSLATPAVRRVAREH
>TRINITY_DN100025_c0_g1_i1
MVENQDGCFFKPGWKEFVRSNDLEGGDFLVFNLVDKISYQVVIFDGTCCPKDLCPFSIMNPIFQHLR
>TRINITY_DN10002_c0_g1_i1
MSDELNQVFQRCREQKRPVFVAFVTAGYPDSEETVDILLGLEAGGADIIELGIPFTDPMVDGKTIQD
>TRINITY_DN100061_c0_g1_i1
MQQVVAKLKAIITKTNVTNENSPVENSSSSTSATSSINNSLHGDLSRVFDNMELESNVSNSSISSNI
```

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Annotation

- Prediction of gene structures in genome sequence
  - Genes are described in text file: Generic Feature Format (GFF)

```
Chr1	TAIR10	chromosome	1	30427671	.	.	.	ID=Chr1;Name=Chr1
Chr1	TAIR10	gene	3631	5899	.	+	.	ID=AT1G01010;Note=protein_coding_gene;Name=AT1G01010
Chr1	TAIR10	mRNA	3631	5899	.	+	.	ID=AT1G01010.1;Parent=AT1G01010;Name=AT1G01010.1;Index=1
Chr1	TAIR10	protein	3760	5630	.	+	.	ID=AT1G01010.1-Protein;Name=AT1G01010.1;Derives_from=AT1G01010.1
Chr1	TAIR10	exon	3631	3913	.	+	.	Parent=AT1G01010.1
Chr1	TAIR10	five_prime_UTR	3631	3759	.	+	.	Parent=AT1G01010.1
Chr1	TAIR10	CDS	3760	3913	.	+	0	Parent=AT1G01010.1,AT1G01010.1-Protein;
Chr1	TAIR10	exon	3996	4276	.	+	.	Parent=AT1G01010.1
Chr1	TAIR10	CDS	3996	4276	.	+	2	Parent=AT1G01010.1,AT1G01010.1-Protein;
Chr1	TAIR10	exon	4486	4605	.	+	.	Parent=AT1G01010.1
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Chr1	TAIR10	CDS	4706	5095	.	+	0	Parent=AT1G01010.1,AT1G01010.1-Protein;
Chr1	TAIR10	exon	5174	5326	.	+	.	Parent=AT1G01010.1
Chr1	TAIR10	CDS	5174	5326	.	+	0	Parent=AT1G01010.1,AT1G01010.1-Protein;
Chr1	TAIR10	exon	5439	5899	.	+	.	Parent=AT1G01010.1
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Chr1	TAIR10	three_prime_UTR	5631	5899	.	+	.	Parent=AT1G01010.1
Chr1	TAIR10	gene	5928	8737	.	-	.	ID=AT1G01020;Note=protein_coding_gene;Name=AT1G01020
Chr1	TAIR10	mRNA	5928	8737	.	-	.	ID=AT1G01020.1;Parent=AT1G01020;Name=AT1G01020.1;Index=1
Chr1	TAIR10	protein	6915	8666	.	-	.	ID=AT1G01020.1-Protein;Name=AT1G01020.1;Derives_from=AT1G01020.1
Chr1	TAIR10	five_prime_UTR	8667	8737	.	-	.	Parent=AT1G01020.1
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Chr1	TAIR10	exon	8417	8464	.	-	.	Parent=AT1G01020.1
```
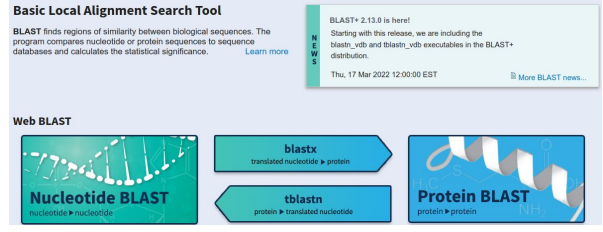

- Assignment of functions to predicted genes
  - BLAST, InterProScan5, Pfam



https://blast.ncbi.nlm.nih.gov/Blast.cgi

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Read mapping

- Alignment of reads to a genome sequence
- Mapping needs to be fast at the cost of accuracy
- Long read mapping tools: minimap2, GraphMap, NGMLR
- Manual inspection of read mappings via Integrated Genomics Viewer (IGV)

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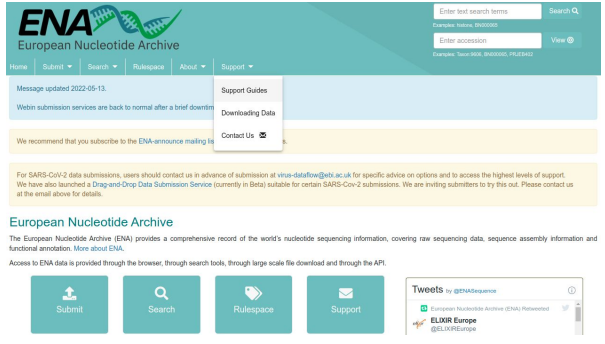
Variant calling

- Identification of sequence differences between reads and reference sequence
- Differences are listed in a specific file type: Variant Caller Format (VCF)
- ONT long reads are well suited for the identification of large structural variants

Databases for submission of sequencing data

- Sequence Read Archive

- European Nucleotide Archive



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Nanopore sequencing trends

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Barcoding

- Multiplexing of different samples in one sequencing run requires individual tags (=barcodes)
- PCR-free barcoding of 12 samples available
- Reads can be separated in real time based on barcodes

‘Read Until’ adaptive sampling / Targeted sequencing

- Check DNA strand based on first sequenced bases against reference:
  - strand is of interest: continue
  - strand is not of interest: reject
  - Example: only sequence bacterial DNA included in a plant sample

- Cas9 Sequencing Kit:
  - Dephosphorylation of 5’-DNA ends
  - Cas9 binds to target sequences (based on guide RNA)
  - Cleavage results in blunt ends and 5’-phosphorylation
  - 3’ dA-tailing to prepare for adapter ligation
  - Adapters are preferentially ligated to Ca9 cut sites

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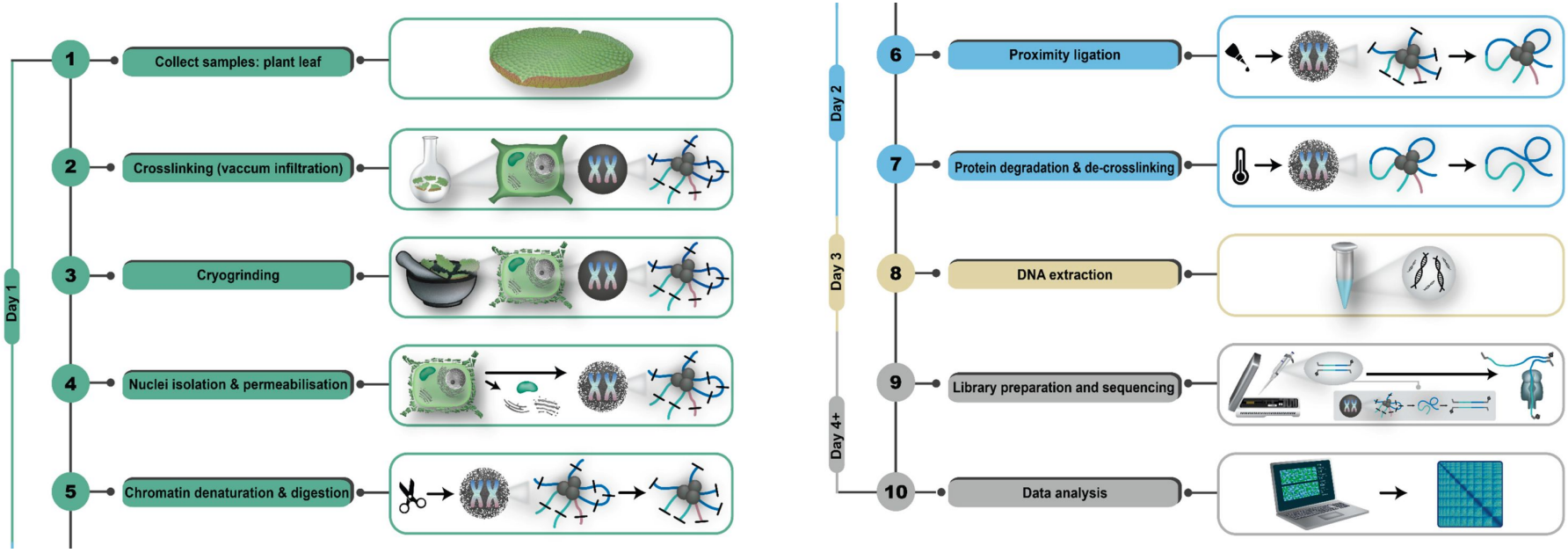
Direct RNA sequencing

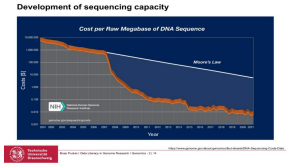
- PolyA tail (3') required for primer ligation
- Generation of complementary DNA strand through reverse transcriptase recommended
- Sequencing of RNA strand allows analysis of base modifications

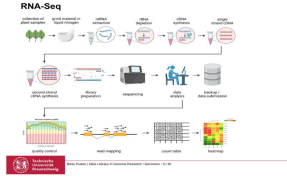
Pore-C (Hi-C with ONT)

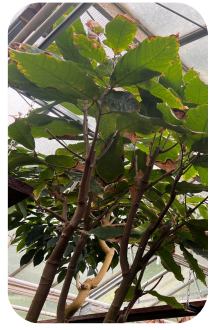


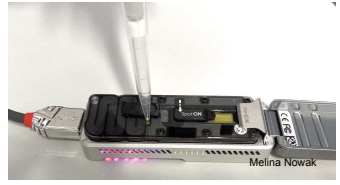
de Oliveira et al., 2025: 10.20944/preprints202508.1176.v2
https://www.protocols.io/view/pore-c-protocol-for-plant-samples-rm7vz9mmrgx1/v1

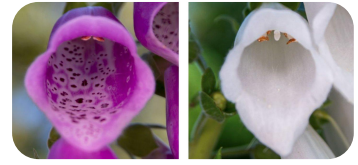
Teaching sequencing in practical courses

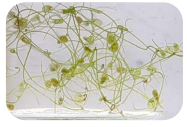












Data Literacy and Plant Genomics

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Genome sequencing projects (examples)

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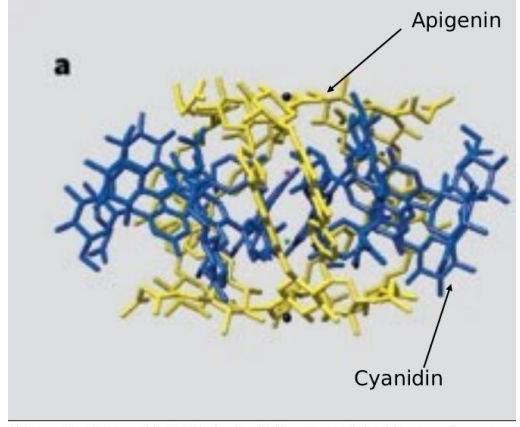
Centaurea cyanus

- Famous for a striking blue flower color
- Complex comprising pigments and metal
- Genome sequence reveals pigment biosynthesis genes



Shiono, M., Matsugaki, N. & Takeda, K. Structure of the blue cornflower pigment. *Nature* **436**, 791 (2005). https://doi.org/10.1038/436791a

SynBio2024 TU Braunschweig
Engineering Blue Flower Colouration

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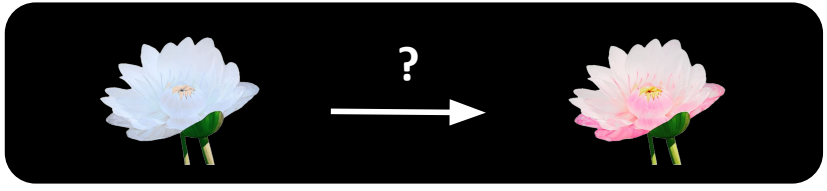
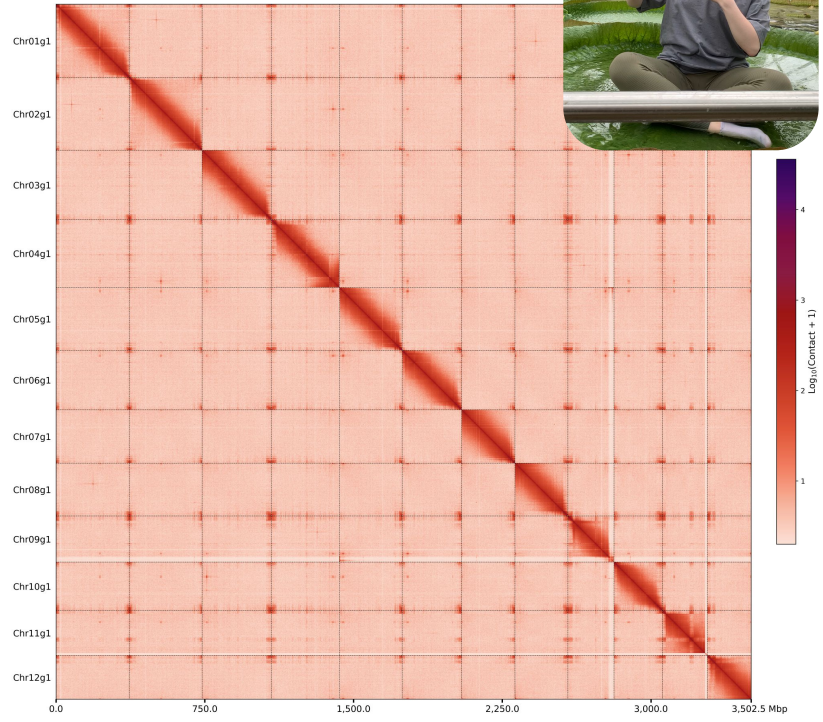
Victoria cruziana

- Water lily with giant floating leaves
- Flowers at night and floral color transitions from white to pink
- Pore-C resulted in an almost perfect genome sequence





Nowak *et al.*, 2025: 10.1101/2024.06.15.599162

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Aquilegia vulgaris

- Popular ornamental plant
- Huge variety of different flower colors
- Genome sequence provides access to pigment biosynthesis genes

Friedhoff et al., 2024: 10.1101/2024.12.16.628782

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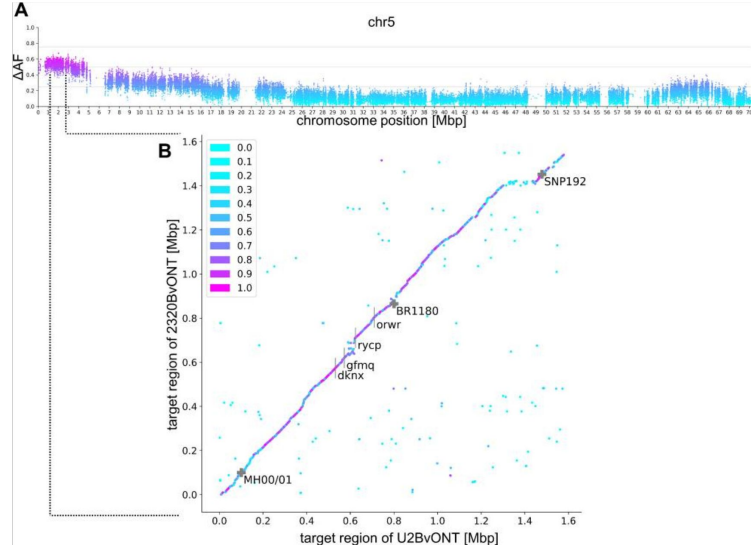
Beta vulgaris

- Important crop in northern hemisphere
- Accounts for about 20% of sugar production
- Nematodes cause serious yield losses



Sielemann et al., 2023: 10.1186/s12864-023-09823-2

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Digitalis purpurea

- Ornamental plant with magenta flowers
- Flower color and shape variation
- Genome sequence reveals underlying genes

Horz *et al.*, 2024: 10.1101/2024.02.14.580303

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Summary

- Planning the sequencing experiment
- DNA extraction, quality control, library preparation
- Nanopore sequencing & real time monitoring
- Data analysis (overview)
- Nanopore sequencing applications
- Genome sequencing projects (examples)

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Time for questions!

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Literature

- de Oliveira, J. A. V. S.; Choudhary, N.; Meckoni, S. N.; Nowak, M. S.; Hagedorn, M.; Pucker, B. (2025). Cookbook for Plant Genome Sequences. doi: 10.20944/preprints202508.1176.v2.

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- Pucker B, Irisarri I, de Vries J and Xu B (2022). Plant genome sequence assembly in the era of long reads: Progress, challenges and future directions. Quantitative Plant Biology, 3, E5. doi: 10.1017/qpb.2021.18.