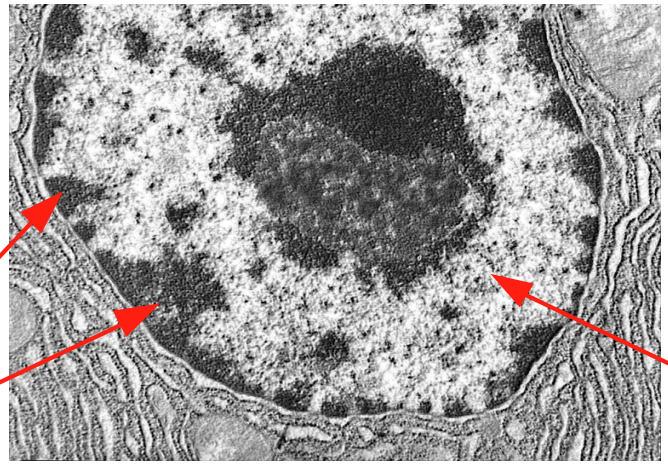
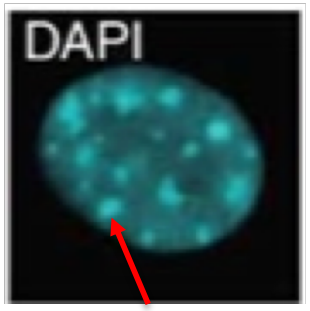
HOW TO STUDY EPIGENETIC MODIFICATIONS

-- STRATEGIES USING SUV39H1 AS A HALLMARK MODEL FOR EPIGENTIC REGUALTION --

Chromatin comes in different flavors

Different types of chromatin





Chromocenter
(aggregates of centromeres
= constitutive heterochromatin)

heterochromatin

euchromatin
(and facultative heterochromatin)

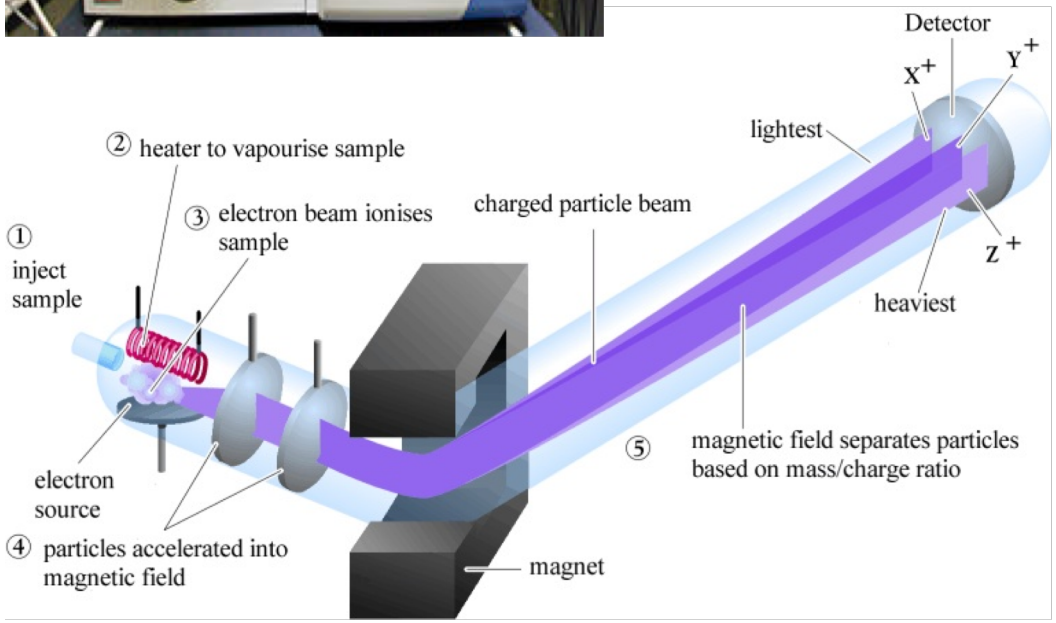
Constitutive heterochromatin:

- constitute ~ 10% of nuclear DNA; telomeres, centromeres, and a considerable fraction of repetitive sequences
- highly compacted, replicates late in S phase, (transcriptionally inert)

Euchromatin + facultative heterochromatin:

- constitute ~ 90% of nuclear DNA
- less condensed, rich in genes, replicates early in S phase
  however,
- only small fraction of euchromatin is transcriptionally active
- the rest is transcriptionally inactive/silenced (but can be activated in certain tissues or developmental stages) → these inactive regions are also known as “facultative heterochromatin”

2. Studying histone modifications by mass spectroscopy



In a typical MS procedure proteins are ionized, for example by bombarding it with electrons. This may cause some of the sample's molecules to break into charged fragments.

These ions are then separated according to their mass-to-charge ratio, typically by accelerating them and subjecting them to an electric or magnetic field:

Ions of the same mass-to-charge ratio (m/z ratio) will undergo the same amount of deflection. Ions with different mass-to-change ratio will show different deflection

→mono-methylated H3K9 has different defection than di-or tri-methylated H3K9

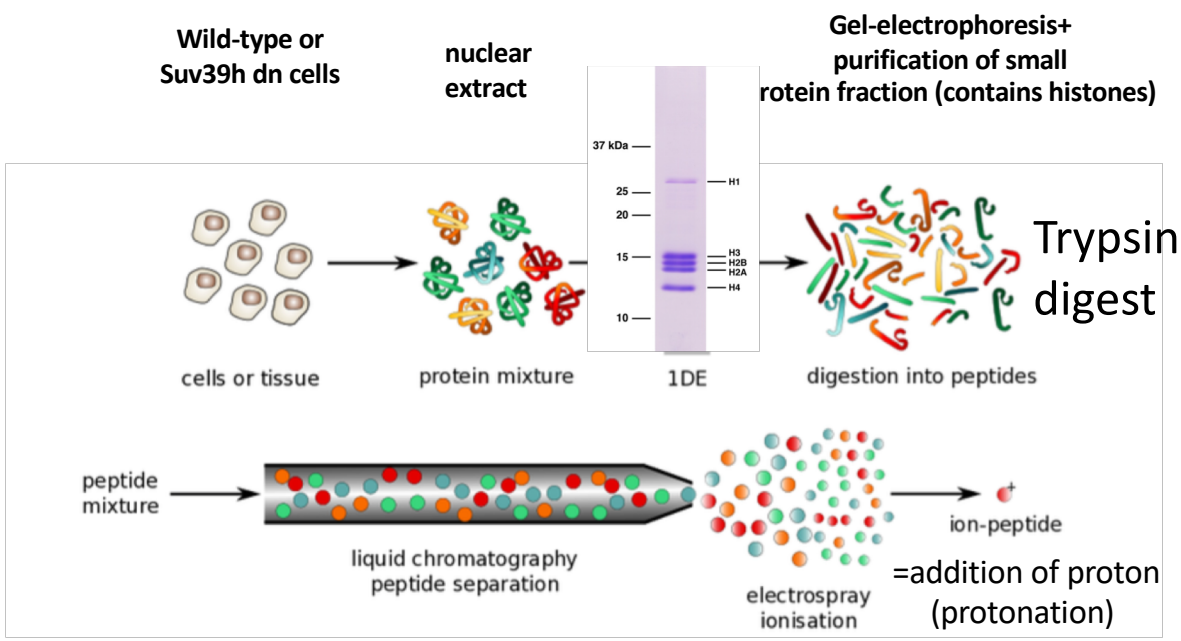
The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio.

The molecules in the sample can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern = **m/z spectrum**.

MW of all amino acids and all their possible modifications are know = identifiable by mass-to-change ration: also when present in a series of aminoacids

MOST IMPORTANT: provide a sample with defined peptide fragments → achieved by digest with proteases that cut peptides at defined positions (comparable with restriction enzymes)

2. Studying histone modifications by mass spectroscopy



Digestion of fraction containing histones with proteases creates a "library" of small peptides that are derived from histones:
Protease= Trypsin
Trypsin cleaves after every Lysine (K) and Arginine (R)

IMPORTANT: we know already All proteins and the amminoacid sequence of all proteins of a cell!!!
That means we can predict all possible small peptide sequences that result from a trypsin cleavage

Highly informative fragments on H3

*Downstream MS analysis can focus on **defined fragmets**! Note: the molecular weight:charge ratio can be predicted/calculated for each fragment and each type of mdification (same holds true for other histones!!)*

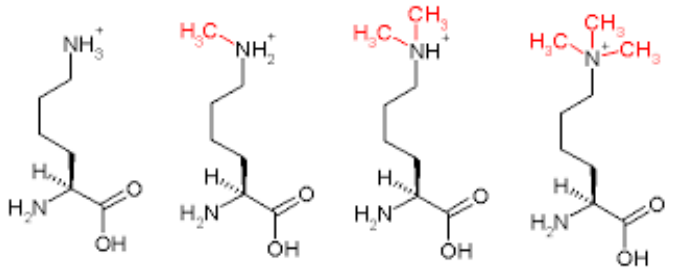
Cutting after K and R

Arows indicate trypsin target sites for cleavage

Histone modifications change m/z ratio in mass spec experiments

Table of amino acid residues .

Symbol	Structure	Mass (Da
Ala A	-NH.CH.(CH_3).CO-	71.0
Arg R	-NH.CH.[$(CH_2)_3$.NH.C(NH).NH_2].CO-	156.1
Asn N	-NH.CH.(CH_2CONH_2).CO-	114.0
Asp D	-NH.CH.(CH_2COOH).CO-	115.0
Cys C	-NH.CH.(CH_2SH).CO-	103.0
Gln Q	-NH.CH.($CH_2CH_2CONH_2$).CO-	**128.1**
Glu E	-NH.CH.(CH_2CH_2COOH).CO-	129.0
Gly G	-NH.CH_2.CO-	57.0
His H	-NH.CH.($CH_2C_3H_3N_2$).CO-	137.1
Ile I	-NH.CH.[CH.(CH_3)CH_2.CH_3].CO-	**113.1**
Leu	-NH.CH.[$CH_2CH(CH_3)_2$].CO-	**113.1**
Lys K	-NH.CH.[$(CH_2)_4NH_2$].CO-	**128.1**
Met M	-NH.CH.[$(CH_2)_2$.SCH_3].CO-	131.0
Phe F	-NH.CH.(CH_2Ph).CO-	147.1
Pro P	-NH.$(CH_2)_3$.CH.CO-	97.1
Ser S	-NH.CH.(CH_2OH).CO-	87.0
Thr T	-NH.CH.[CH(OH)CH_3).CO-	101.0
Trp W	-NH.CH.[CH_2.C_8H_6N].CO-	186.1
Tyr Y	-NH.CH.[(CH_2).C_6H_4.OH].CO-	163.1
Val V	-NH.CH.[$CH(CH_3)_2$].CO-	99.1



KSTGGK: MW= 577 g/mol CH_3 : MW= 15 g/mol

Kme_3STGGK: MW=577 – 3 + 45 = 619 g/mol

Kme_2STGGK: MW= 577 – 2 + 30 = 605 g/mol

Kme_1STGGK: MW= 577 – 1 + 15 = 591g/mol

Protonation changes MW in a defined manner

Molecular weight

ionization

For details: An Introduction to Mass Spectrometry
http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm

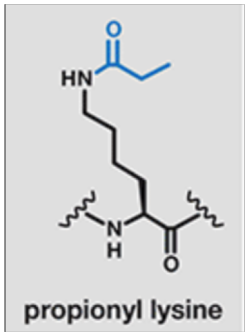
2. Studying histone modifications by mass spectroscopy

PROBLEM:

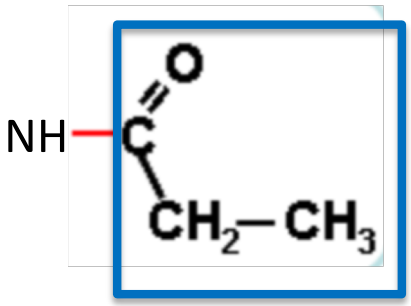
1.Trypsin cleavage after K is not complete because me2 or me3 on some lysines block block cutting by Trypsin

2. K is frequent in histone tails --> result many cleavages (mostly at me0, me1) → many small heterogeneous peptides → difficult to analyze

Trypsin can cleave after Arg (R)
Trypsin can cleave after K-unmethylated; K-me1
Trypsin cannot cleave after K-me2; K-me3

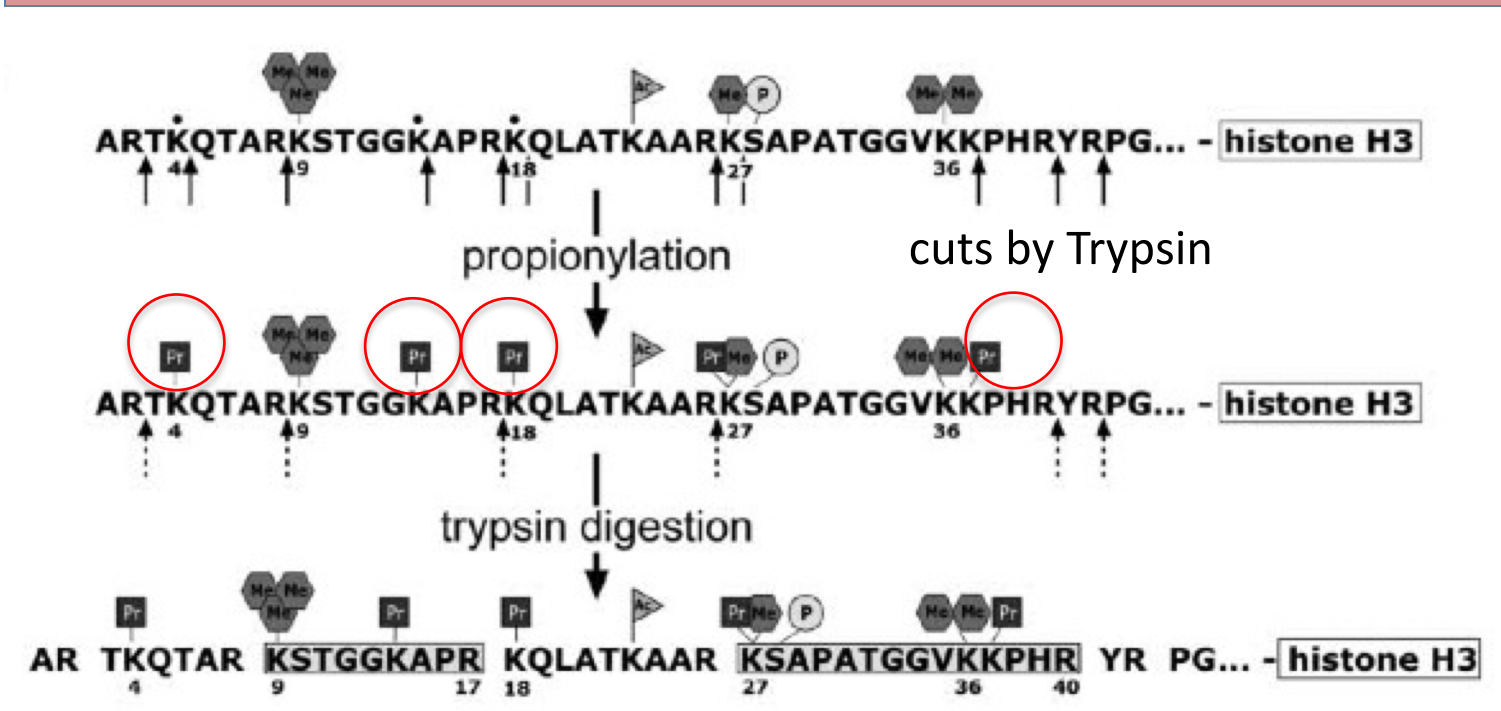


Proprionyl group



SOLUTION: In vitro Propionylation of unmodified or mono-methylated lysine prevents trypsin cleavage
→ now Trypsin can only cut after Arginine.
→ this allows a uniform cleavage of histone tails
→ creates a mass:charge ratio that allows to differentiate between fragments carrying me0, me1, me2 or me3 marks (me0 + 3x propionyl groups; me1 + 2x propionyl groups; me2 + 1 propionyl group; me3 + 0x propionyl groups)

2. Studying histone modifications by mass spectroscopy



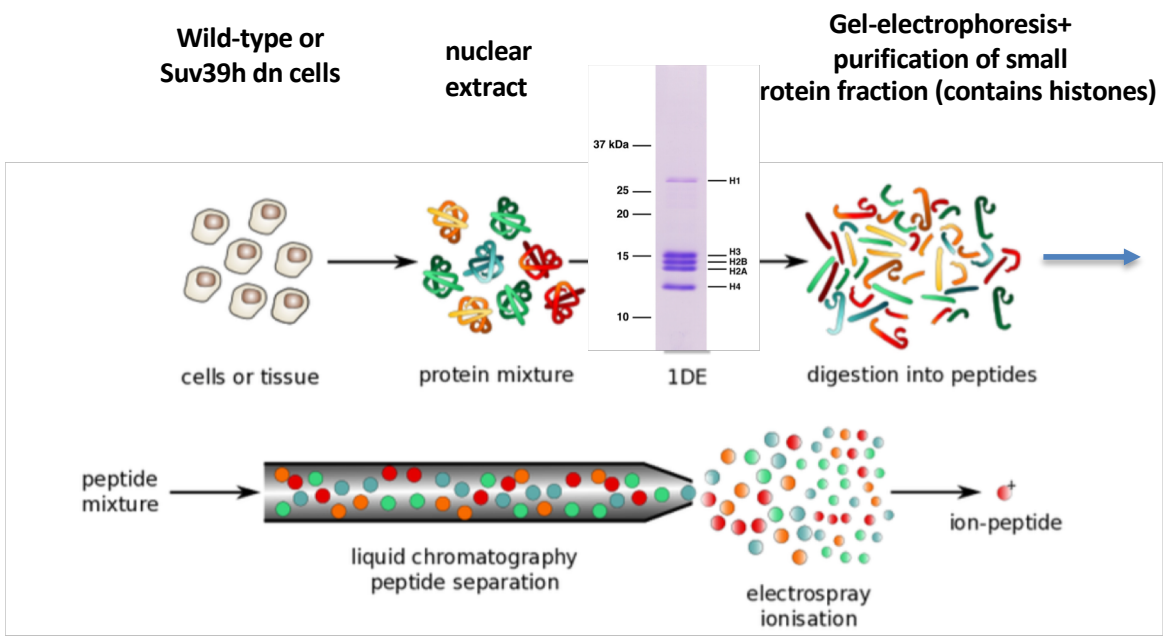
propionylation

bigger and homogeneous fragments

TRYPSIN cuts ONLY at Arg by Trypsin after propionylation

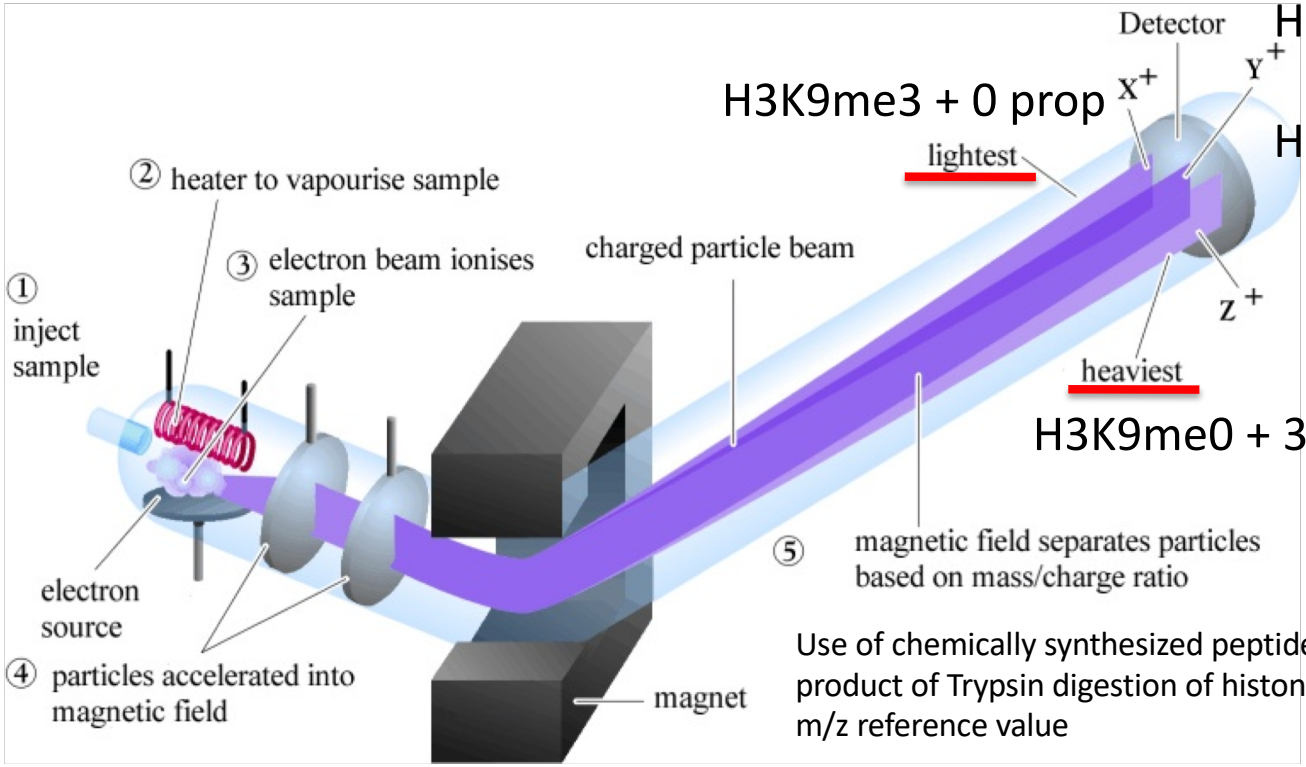
Now Trypsin can only cut after Arginine. This allows a uniform cleavage of histone tails
The number of methyl-groups/propinyl groups at the H3 peptide K9 – R17 decides on the deflection of this specific peptide in mass spectrometry.
Important: All peptides cut at Arg by Trypsin are analyzed contemporarily!!!!

2. Studying histone modifications by mass spectroscopy



Propionylation → ***Trypsin digest***

2. Studying histone modifications by mass spectroscopy

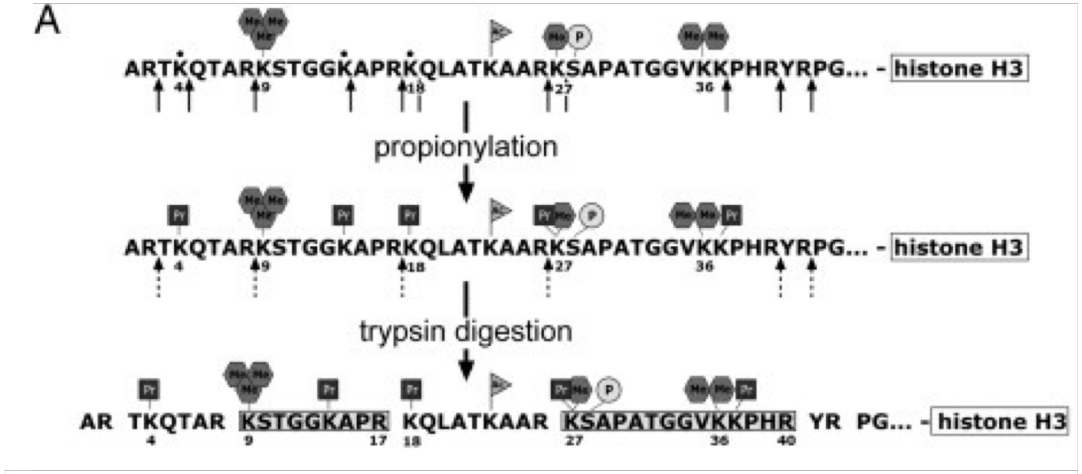


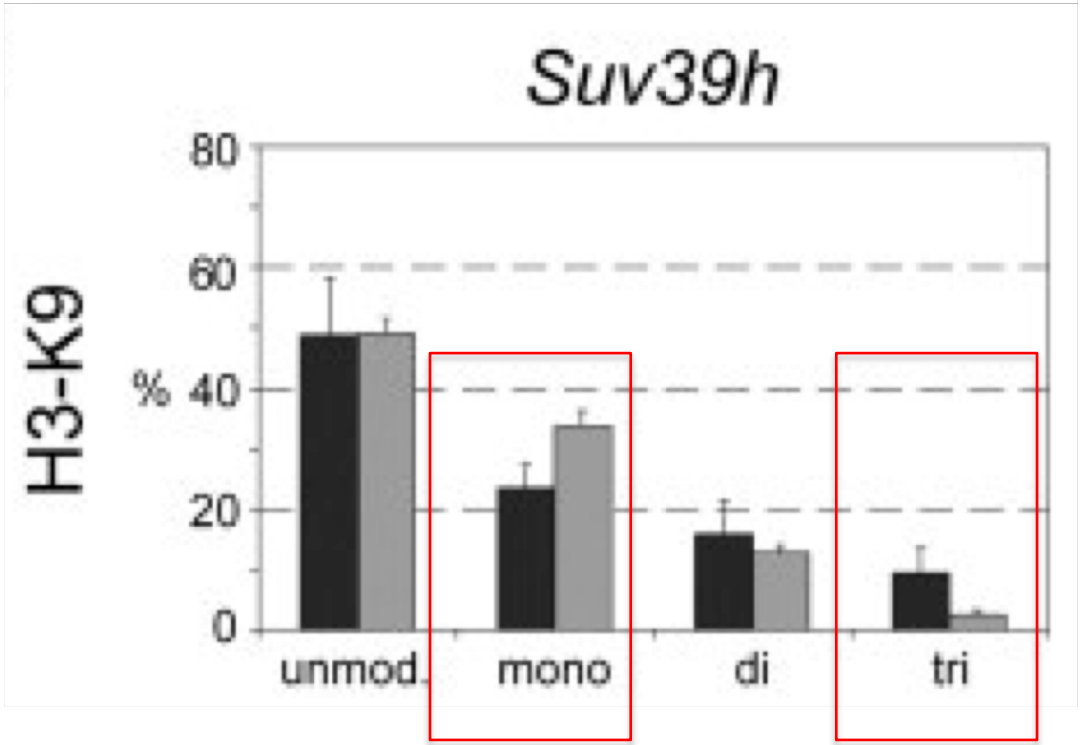
Use of chemically synthesized peptides that reflect the product of Trypsin digestion of histones can be used to have a m/z reference value

The method allows to quantify the numbers of H3K9me0, H3K9me1 H3K9me2 and H3K9me3 in a sample → We can calculate the % of each histone modification in the given sample.

Important: The analysis is not limited to H3K9 → other histone modifications can be quantified in the same analysis

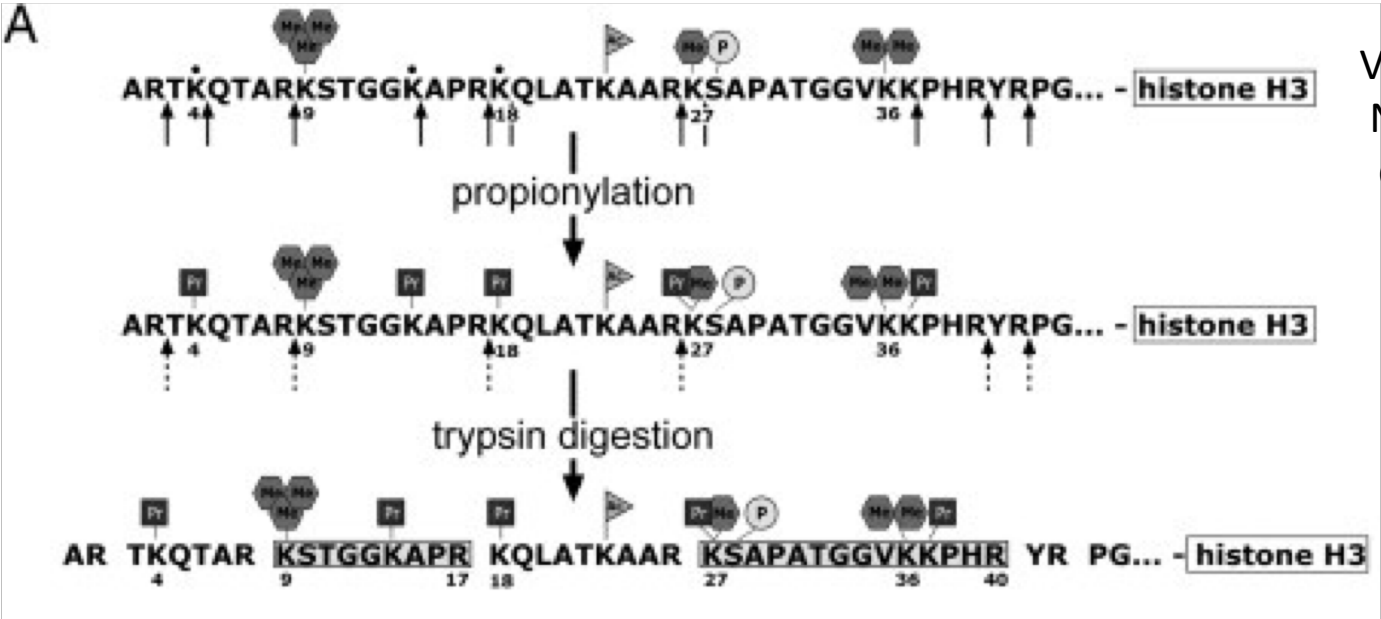
2. Quantifying histone modifications by mass spectroscopy





Suv39h1 and Suv39h2 ensure H3K9me3.
H3K9me1 is a preferred substrate for Suv39h1/h2
Suv39h1/h2 methylate H3K9me1 Until reaching the tri-methylated state

2. Quantifying histone modifications by mass spectroscopy



Very short peptides, Non homogenouse digest - difficult to analyse in MS

Propionylation of unmodified Ks

Propionylation of unmodified Ks

1. Identifying the enzymatic activity of a histone modifying enzymes

2. Identification of “modifiable” amminoacids in histone tails

3. Functional link between histone modifications

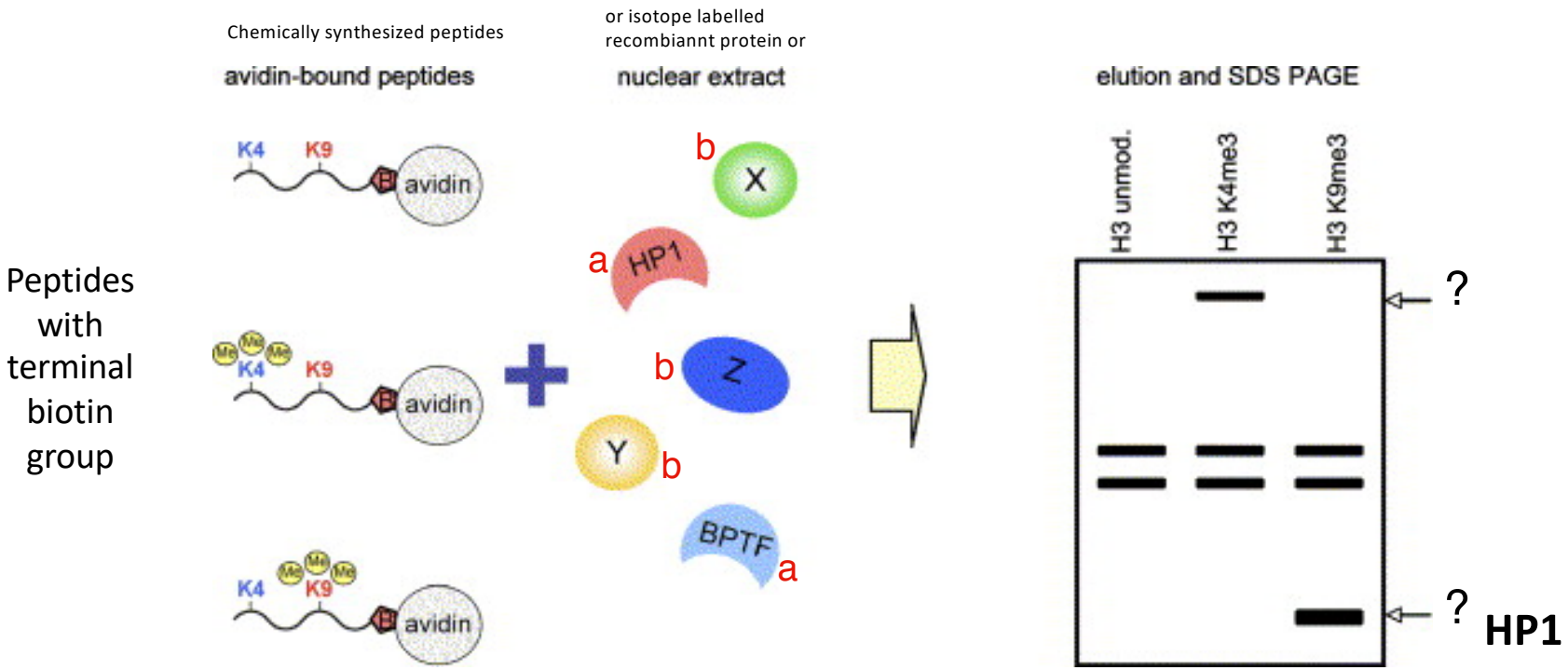
4. Defining the ENTIRE epigenetic status of a cell type (for example: differentiated/stem cell)

5. Quantitative information on histone modifications (%)

HOW CAN EPIGENTIC READERS BE IDENTIFIED?

Identifying of methyl-H3 binding proteins (HP1)

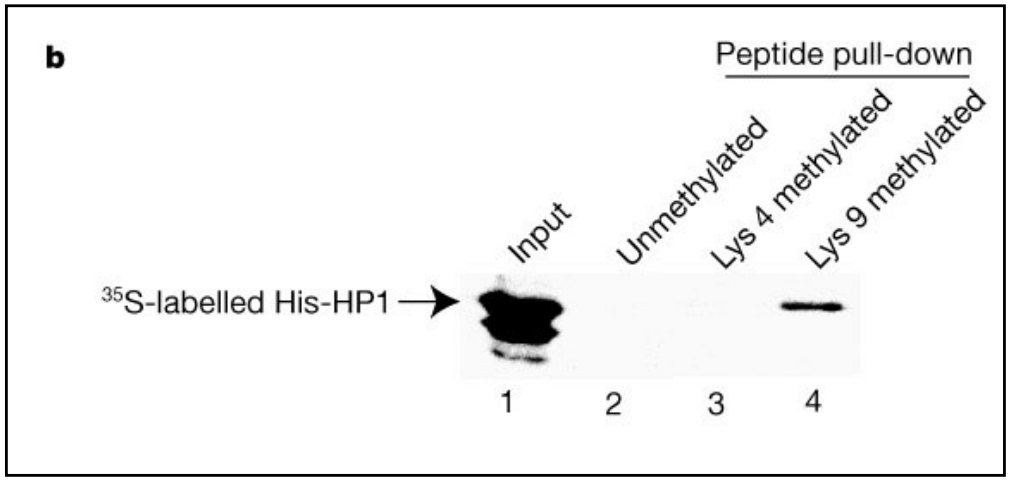
- histone peptide pulldown assay:



a = candidate approach → identify by Western blotting
b = unbiased approach → color gel to locate protein bands, cut out band and identify protein by Mass Spec

HOW CAN EPIGENTIC READERS BE IDENTIFIED???

• Using the peptide pull-down assay, it was found that Lys9-methylated H3 binds to heterochromatin protein 1 (HP1)



Pull-down assay:
-Couple modified histone tail-peptides (methylated, or unmethylated) to resin
-Incubate with recombinant ^{35}S-labelled HP1
-Wash resin
-Elute bound proteins, run gel and make radiography

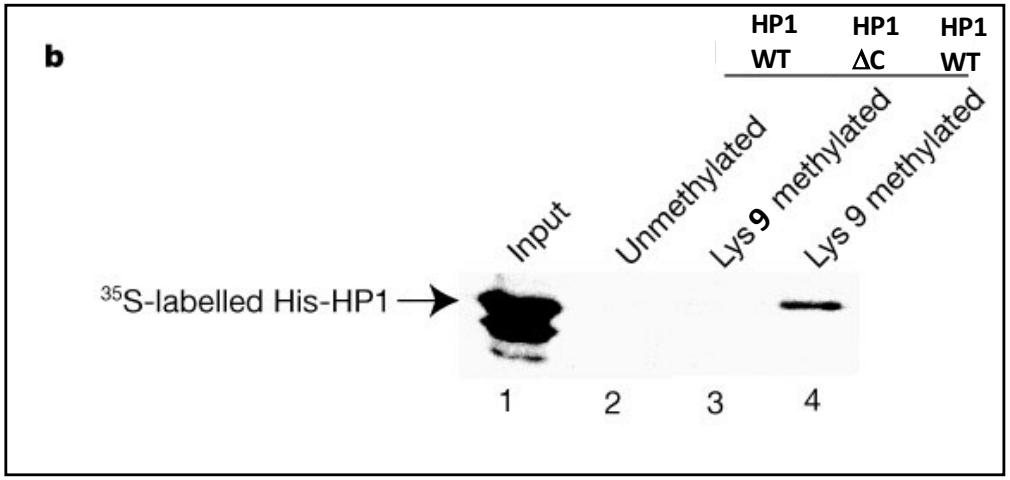
• HP1 is a protein previously identified to be enriched at heterochromatin and important for heterochromatin assembly → now we know that it is directly linked with H3K9me3

What part of HP1 is important for interaction with H3K9me3?

Bannister et al, Nature, 2001

HOW CAN FUNCTIONAL DOMAINS OF EPIGENTIC READERS BE IDENTIFIED???

- What protein domain is interacting with H3K9me?



Pull-down assay combined with deletion analysis:

-Couple modified histone Tail-peptides (methylated, or unmethylated) to resin

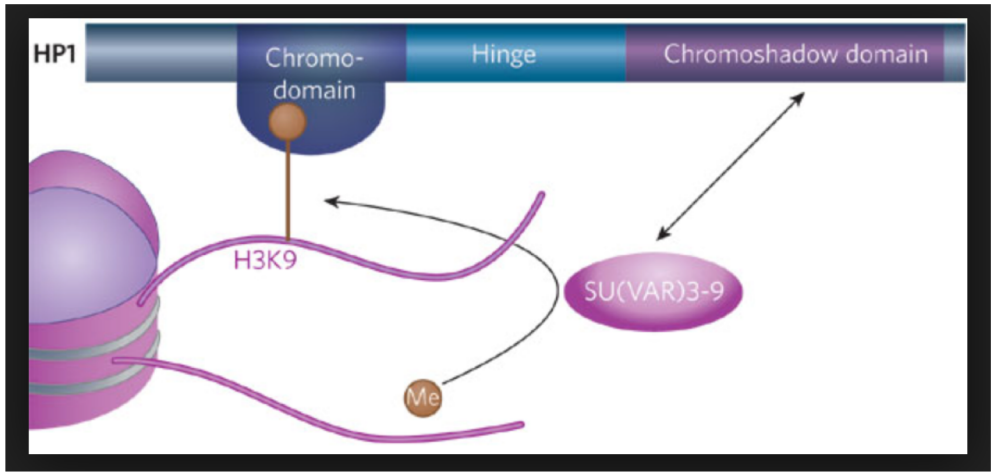
-Incubate with recombinant

-^{35}S-labelled mutant versions of HP1

–ΔC; ΔCS; ΔH

-Wash resin

-Elute bound proteins, run gel and make radiography

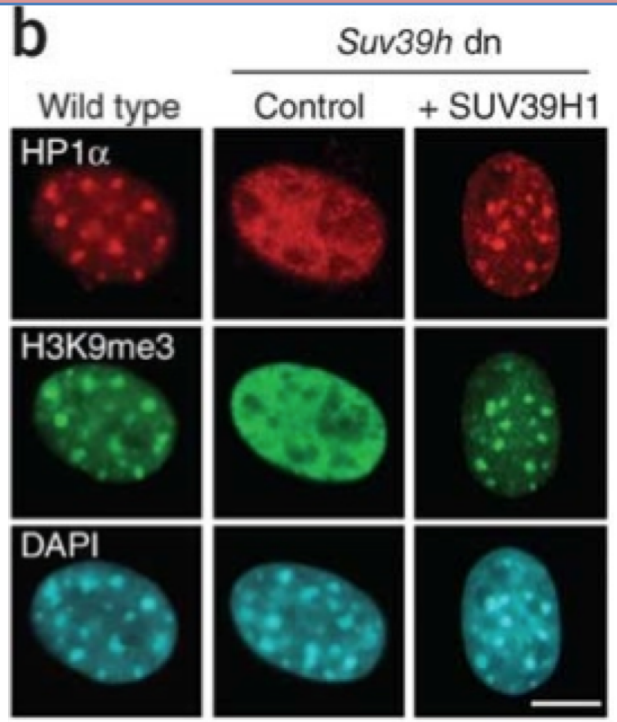


Lys9-methylated H3 binds to the conserved motif called chromodomain

Bannister et al, Nature, 2001

Where does Suv39h1 act in a cell ???

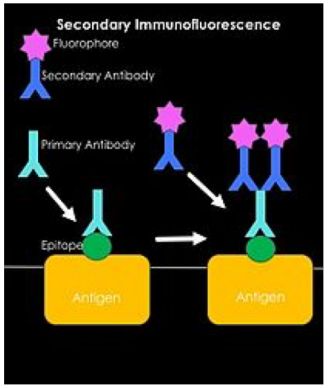
IMMUNOFLUORESCENCE → MACROSCOPIC ANALYSIS
→ Localization of protein across large regions of DNA



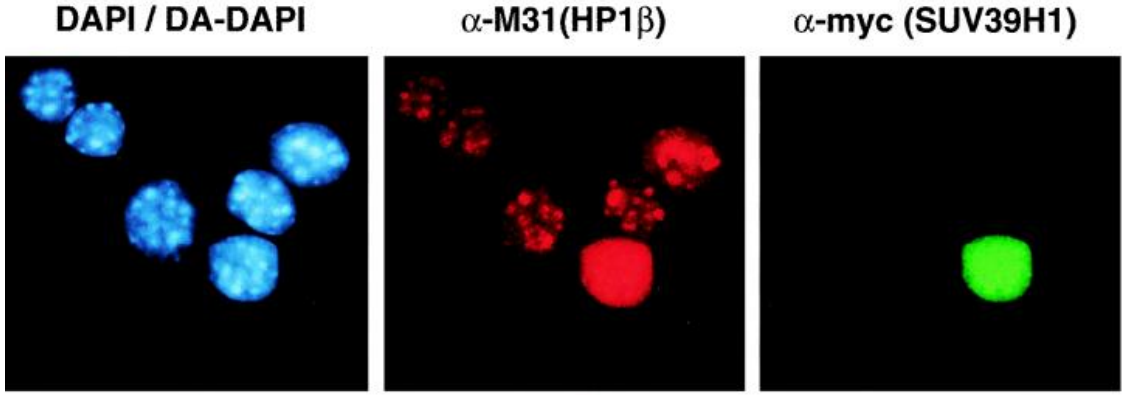
Loss of Suv39h1/h2 causes delocalization Of HP1 from chromocenters

Chromocenters are subnuclear regions where Multiple centromeres aggregate

Chromocenters are DAPI intense regions and represent tightly packed heterochromatin



Co-immunofluorescene (exicatiation with UV light):
Primary, mouse anti-HP1 + secondary rabbit anti-mouse-Cy3 (emission at 570 nm)
Primary, human anti-H3K9me3 + secondary donkey anti-human Alexa 488 (emission at 488 nm)
DAPI (emission 461 nm)

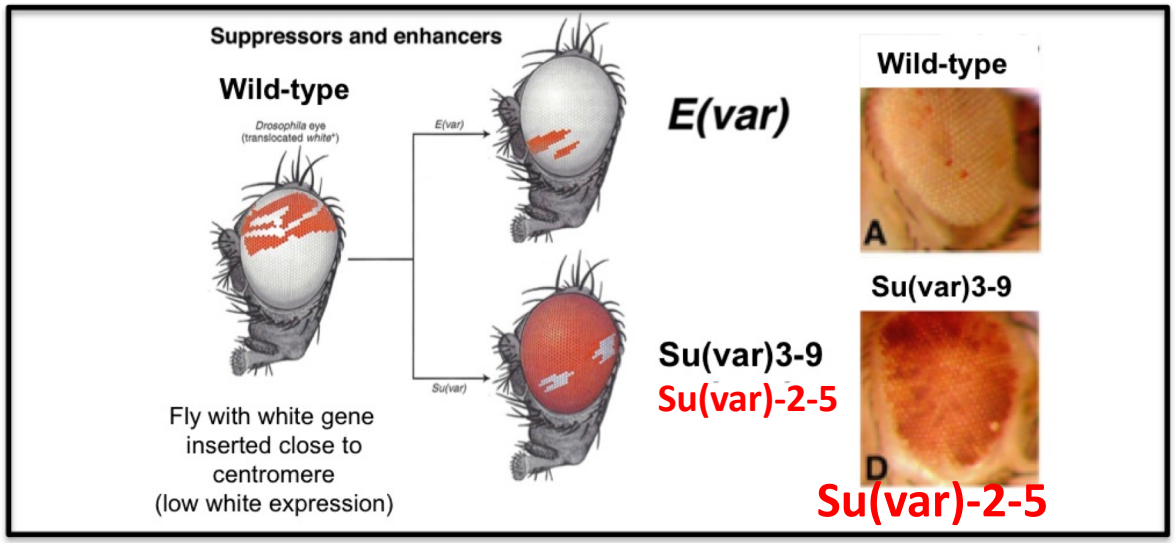


Over-expression of SUV39H1 in Hela cells causes an accumulation of HP1. Stabilization? Is there a link between Suv39h1 – H3K9me3 and HP1??

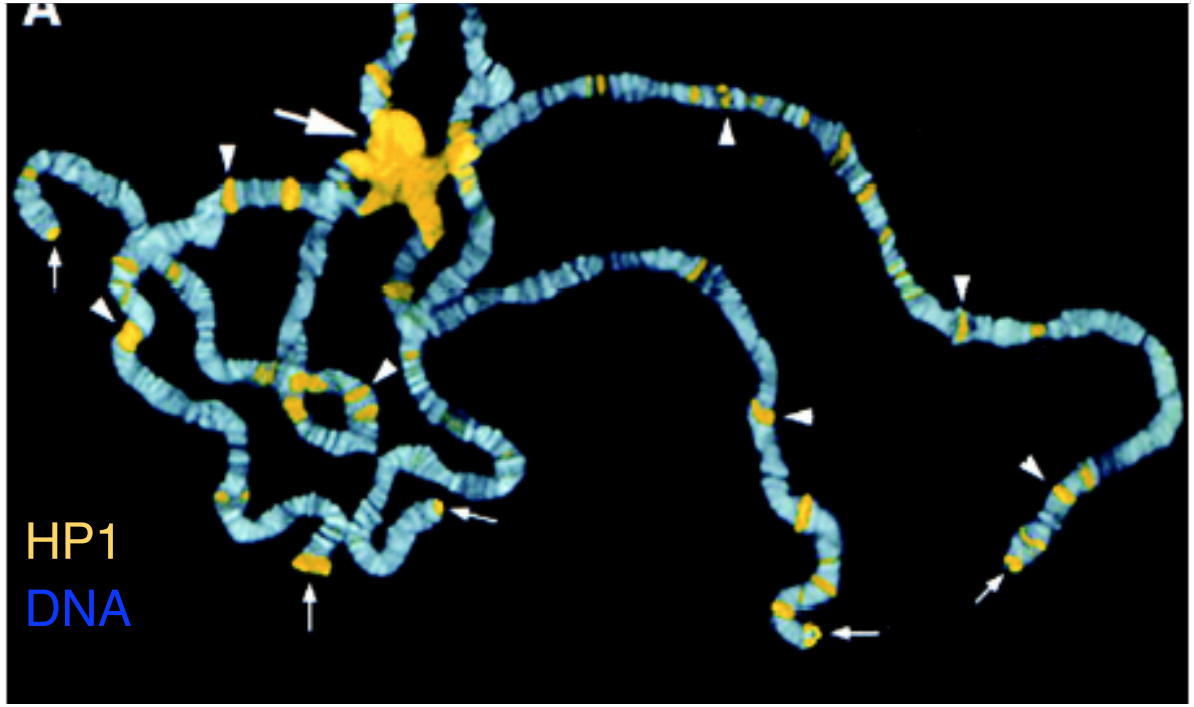
myc-SUV39H1: myc-epitope tagged Suv39H1

Melcher et al, MCB, 2000

What are the target sites for Suv39h1 and H3K9me???

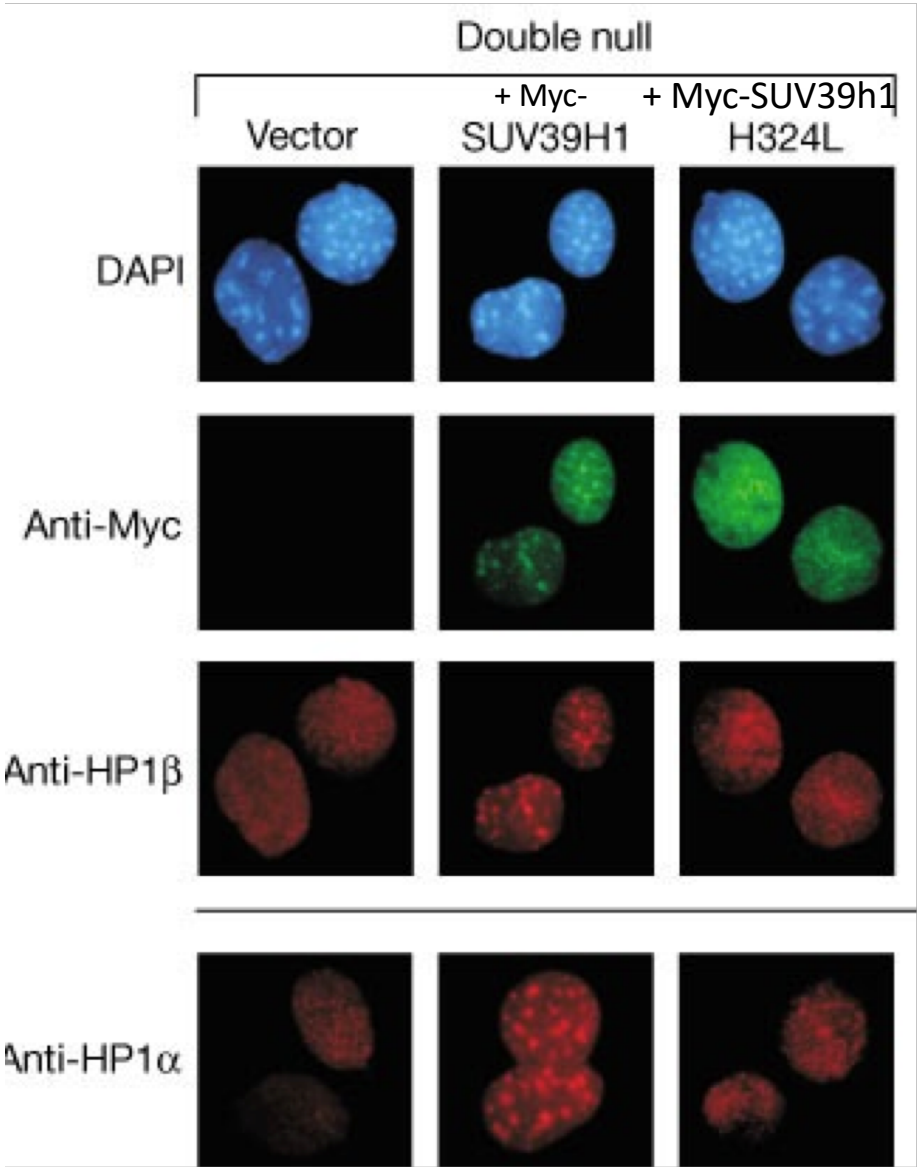


Another evidence for HP1 and Suv39h interaction came from Drosophila



- back to early genetics studies in *Drosophila*:
- Su(var) 2-5 (gene) codes for heterochromatin protein 1
- Su(var) 2-5 shows similar phenotype like Su(var)3-9
- HP1 in Drosophila is localized to the chromocenter

SUV39h HMTase activity is important to build constitutive heterochromatin



Loss of Suv39h1 expression
results in delocalization of
HP1 from chromocenters

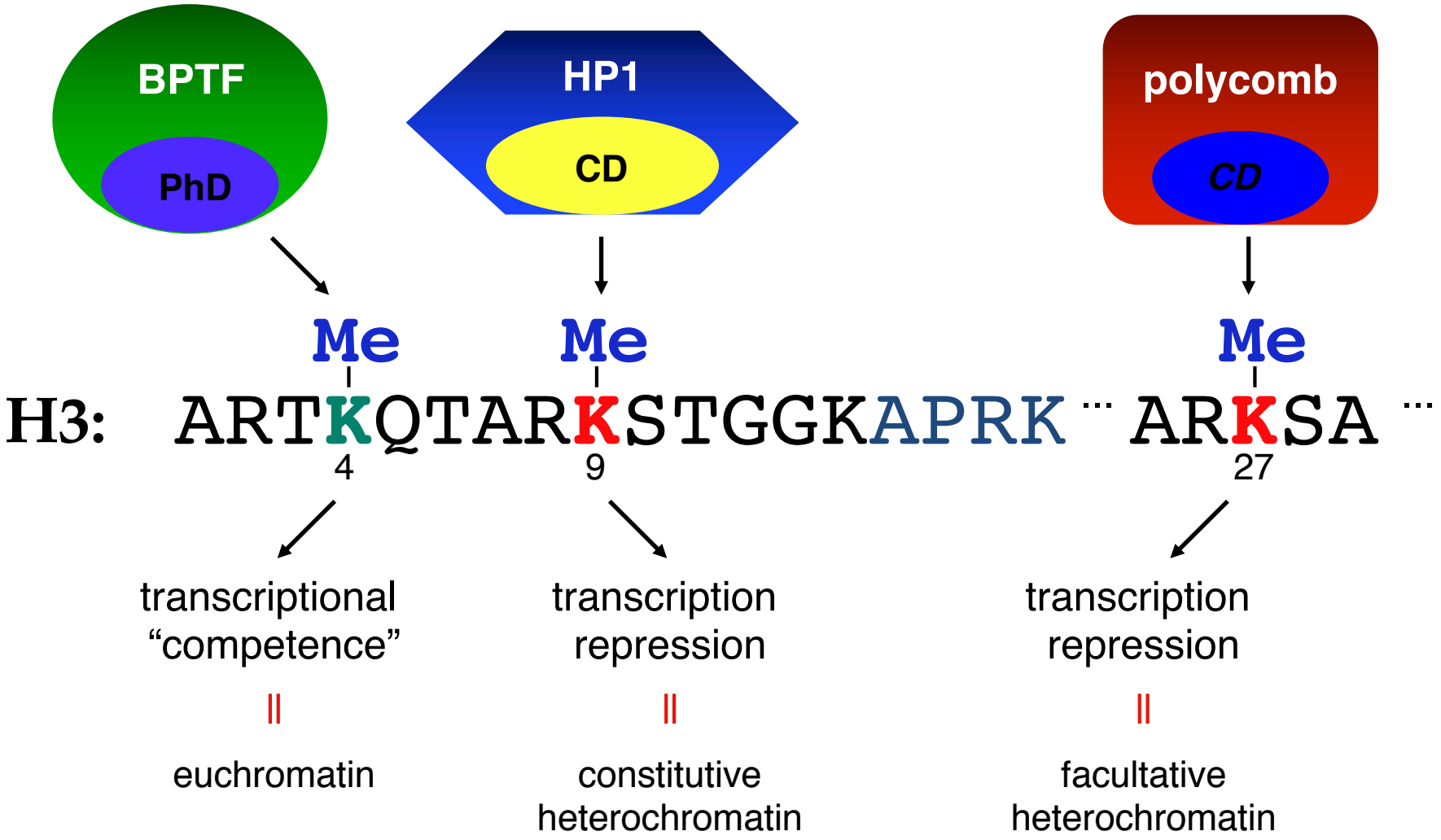
Re-expression of Suv39h1 rescues
Localization of HP1 to chromocenters

Re-expression of Suv39h1 that
Contains an enzymatic dead mutation
(H324L) in SET domain does not rescue the
localization of HP1 to chromo centers

→The enzymatic activity of Suv39h1
Is required for recruitment of HP1
to chromocenters

Lachner et al, Nature, 2001

Site specific methylation of the H3 tail has different functions

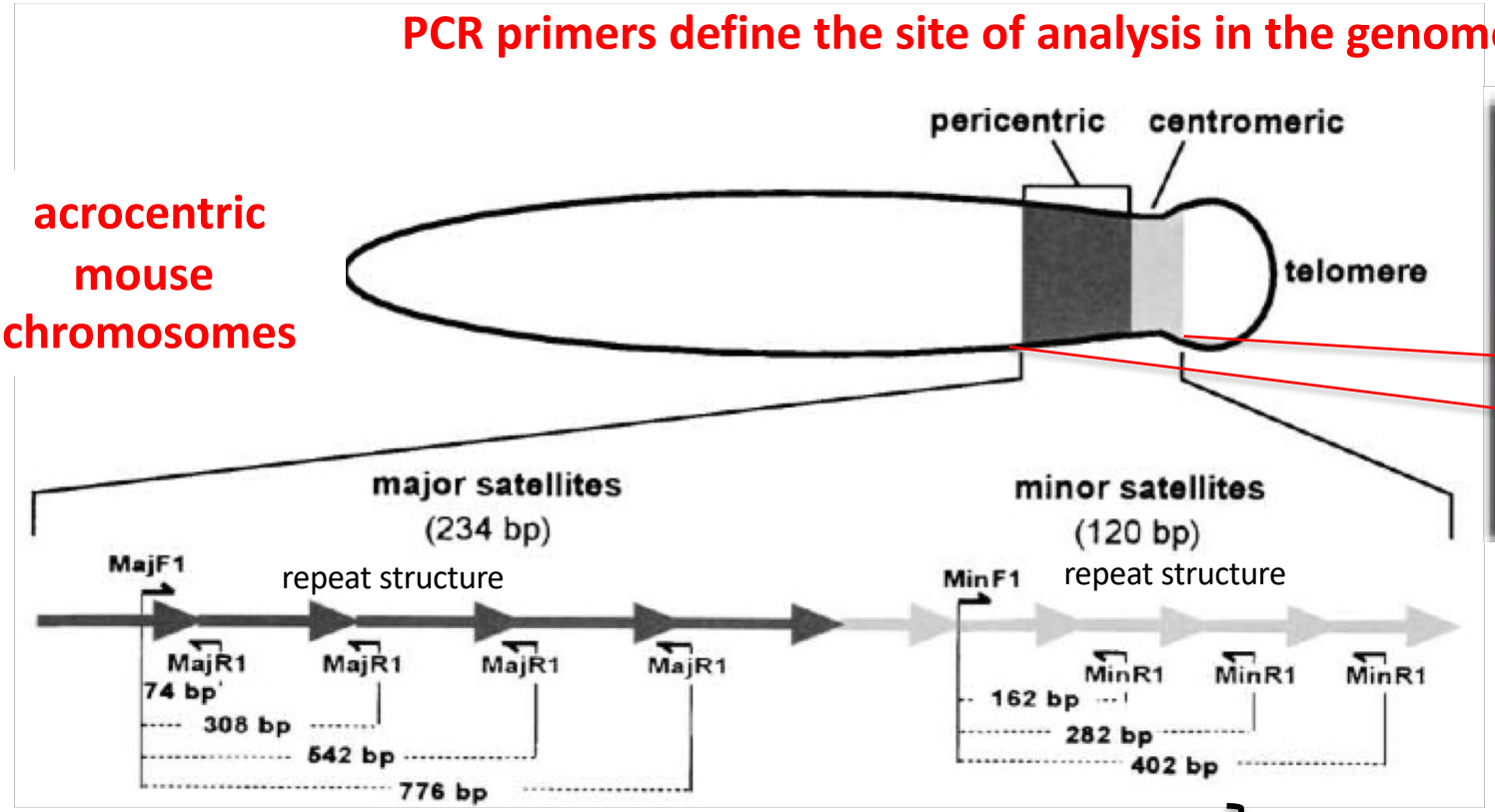


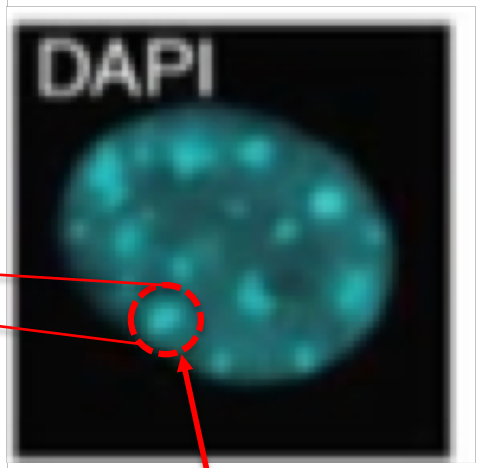
What are the target sites for Suv39h1 and H3K9me???

CHROMATIN IMMUNOPRECIPITATION → DETAILLED ANALYSIS
→ Localization of protein at a defined region - seqeunce

The combination of Immunoprecipitation methods and PCR analysis allows to define the histone code at defined sequences.
PCR primers define the site of analysis in the genome

acrocentric mouse chromosomes





Chromocenter
=centromeric
Regions from
several chromosomes

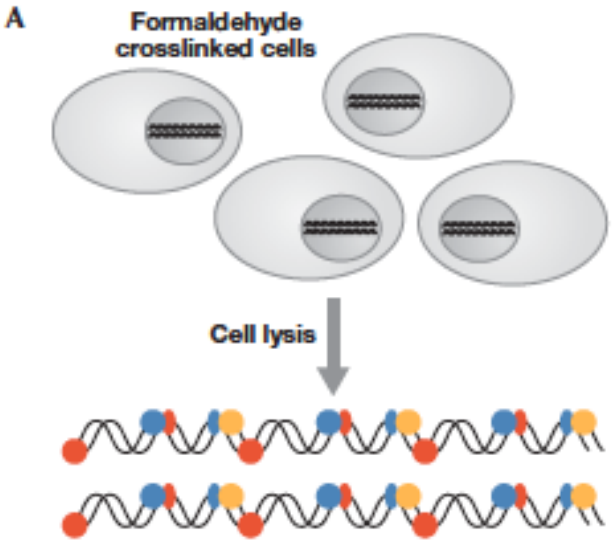
Forward primer: unique
Reverse primer: binds single site in every repeat

} multiple PCR amplicons containing 1, 2, 3 or more repeats

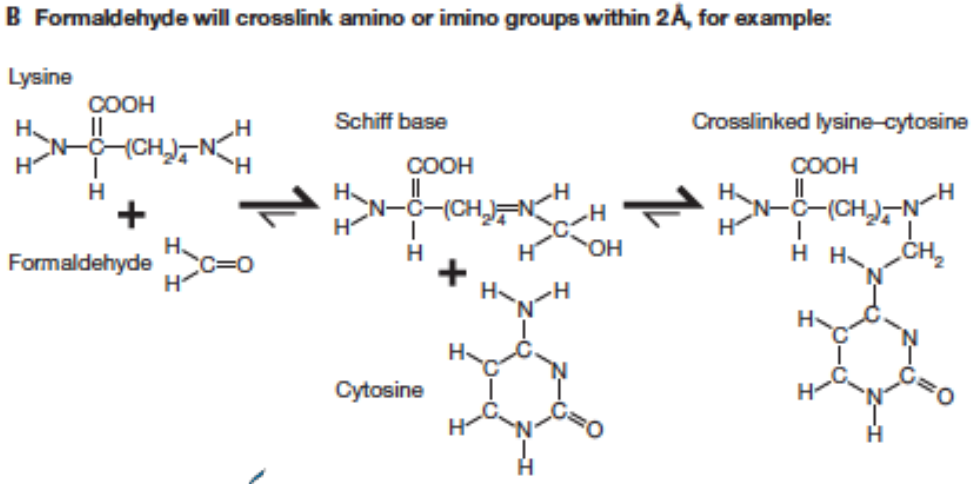
EXAMPLE: Pericentric heterochromatin in mouse cells

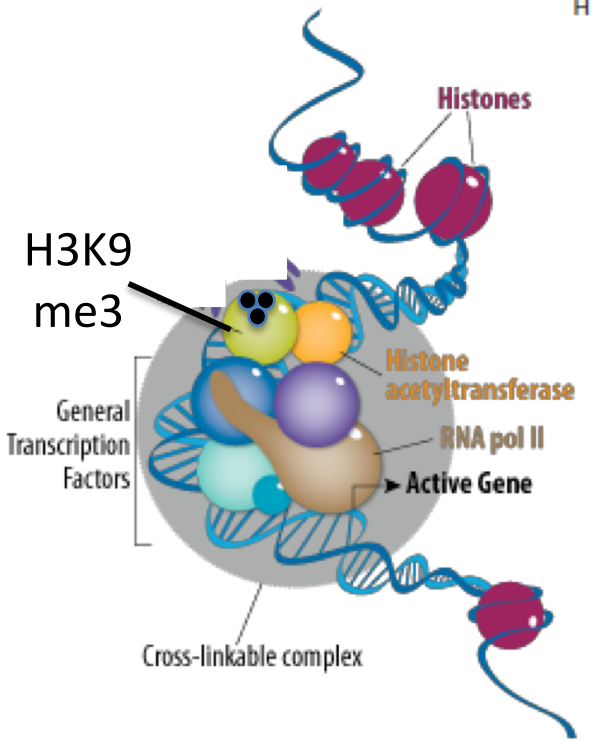
CHROMATIN IMMUNOPRECIPITATION

1. Cross linking with FA



Optimization is crucial.

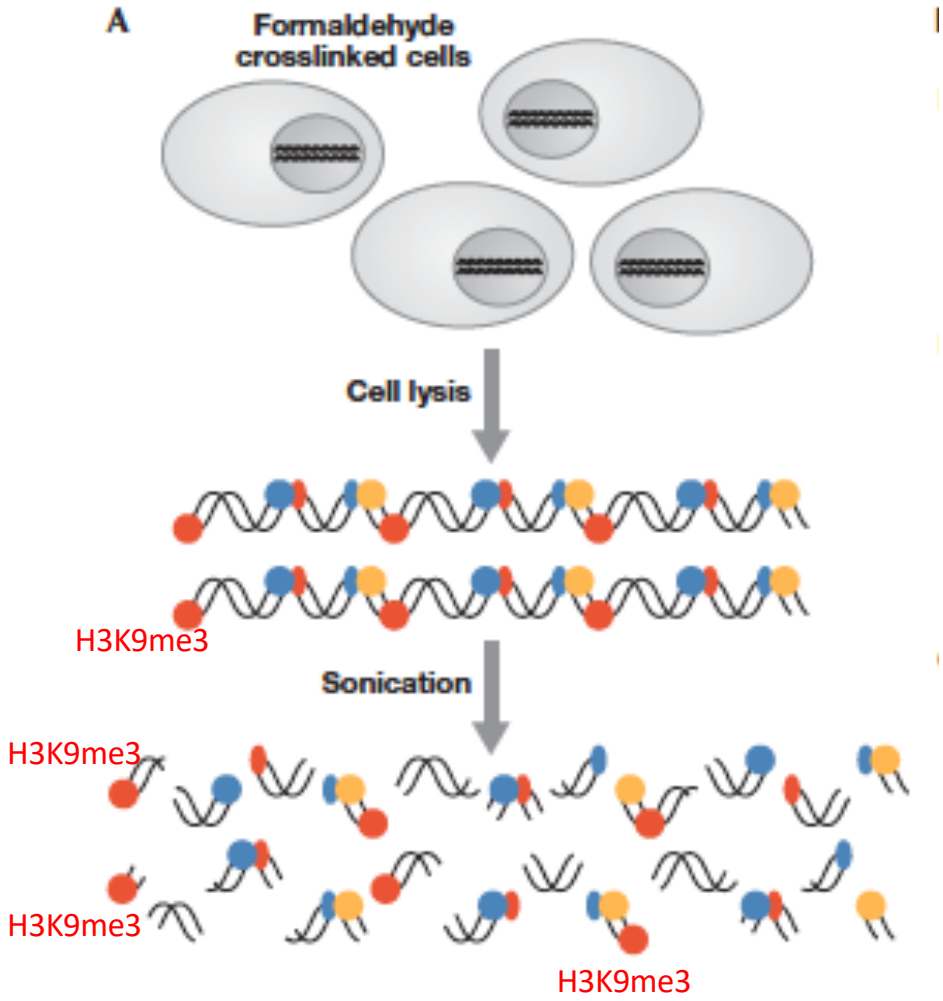


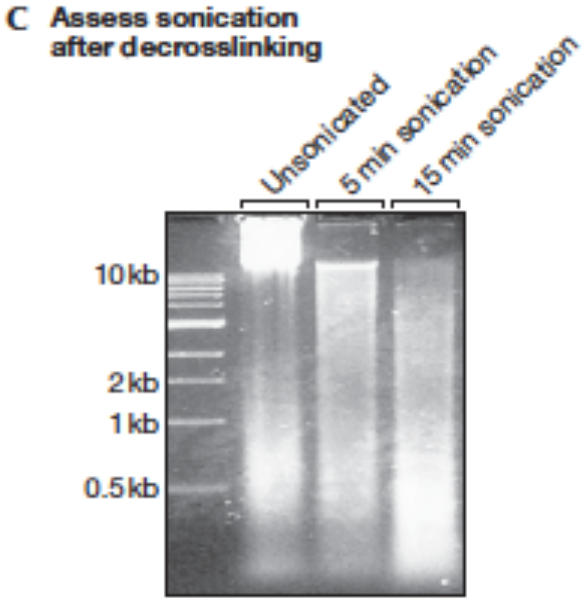


Covalently crosslinked chromatin

CHROMATIN IMMUNOPRECIPITATION

2. Cross linking followed by sonication (fragmentation of chromatin)



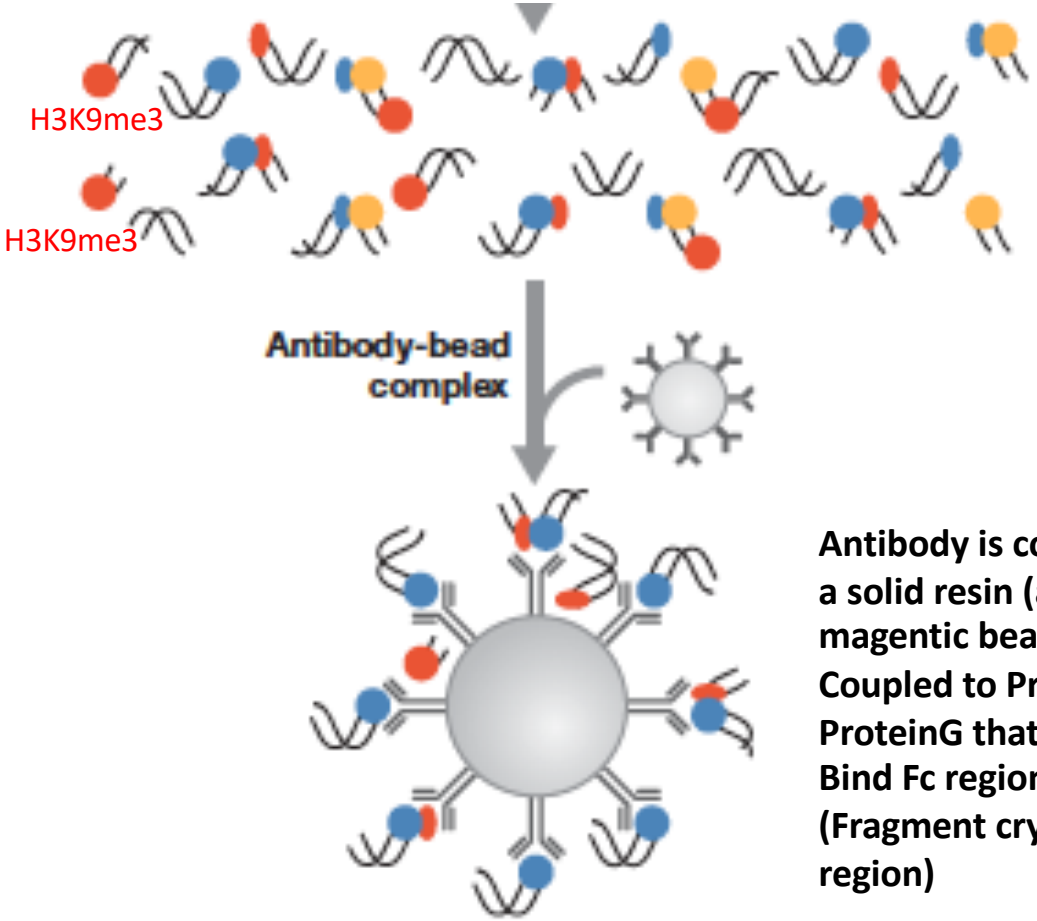


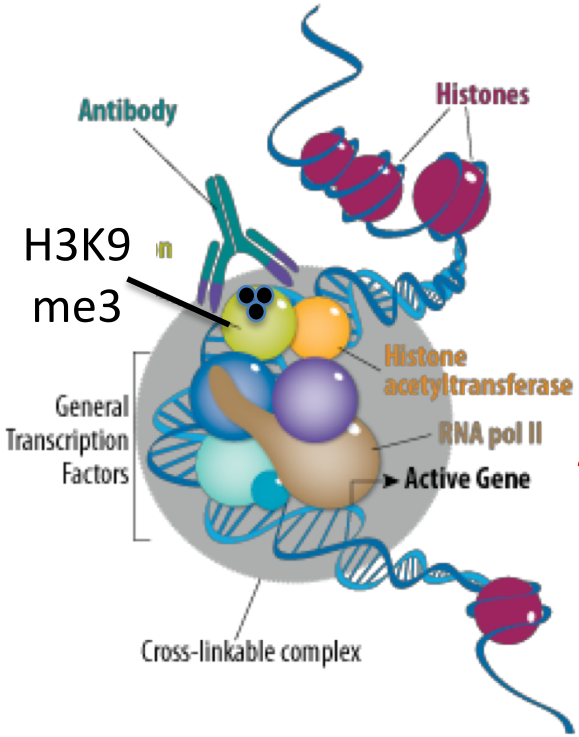
Chromatin is bulky → needs to be cut into small pieces to become soluble

CHROMATIN IMMUNOPRECIPITATION

3. Immunoprecipitation (IP)

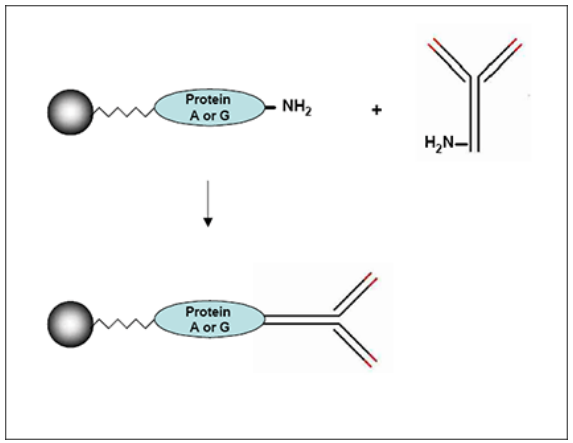
The protein of interest is immunoprecipitated together with the crosslinked DNA: Modified histones; epignetic writers, epigentic readers





Covalently crosslinked, fragmented Chromatin + Anti-H3K9me3

Antibody is coupled to a solid resin (agarose, magentic beads)
Coupled to ProteinA or ProteinG that
Bind Fc region of antibody (Fragment crystallizable region)



CHROMATIN IMMUNOPRECIPITATION

4.Decrosslinking of PFA crosslinked chromatin and and purification of the DNA

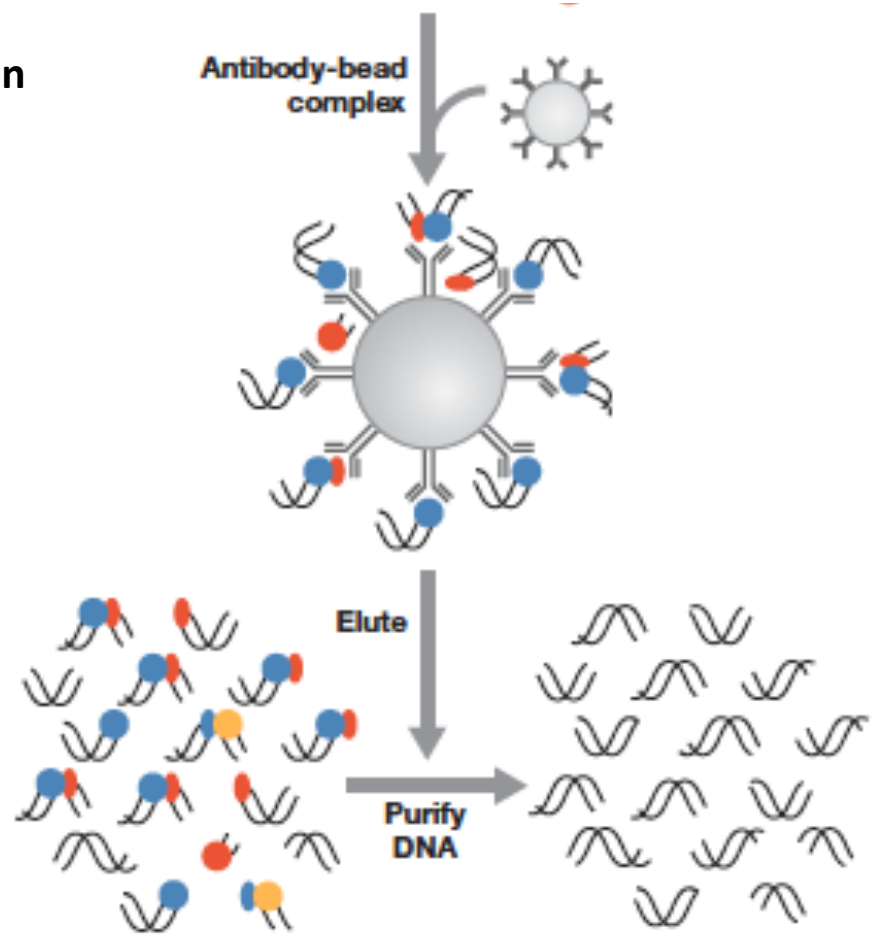
-Recover beads by centrofugation

- Wash beads

- Reverse the FA: 65C, high salt concentrations: crosslink break

- RNAse and Protease treatment

- Purification of DNA

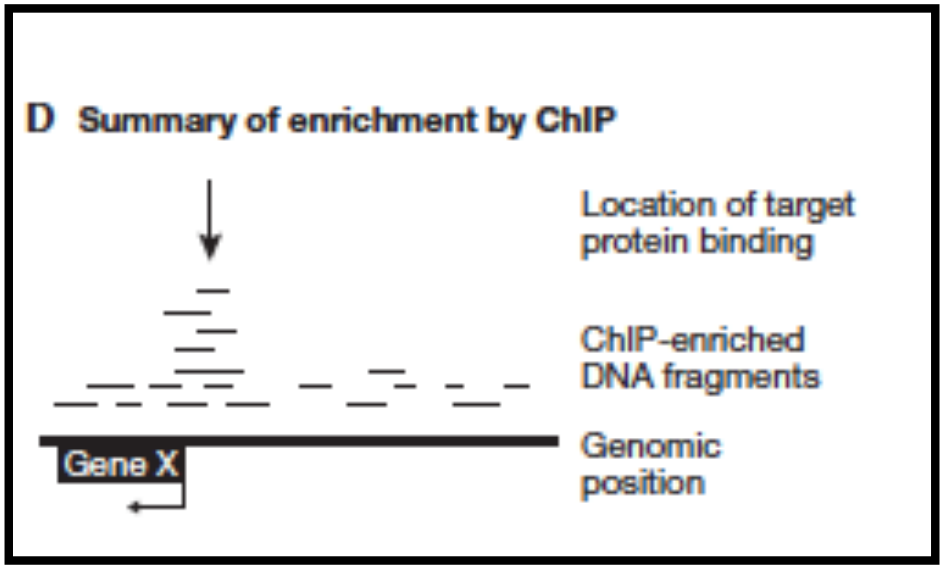


ANALYZE DNA TO IDENTIFY REGIONS WITH HIGH H3K9me3 (sequencing, hybridzation, PCR with gene specific primers

CHROMATIN IMMUNOPRECIPITATION

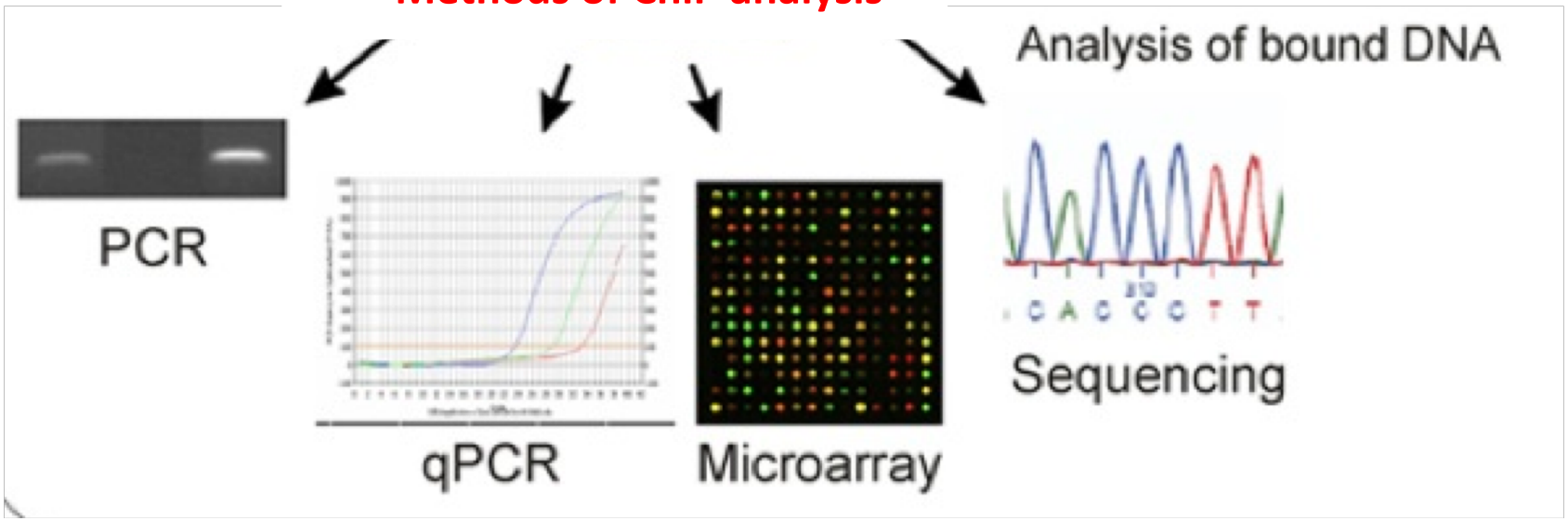
5. Analysis of ChIP DNA

Identification of DNA regions associated with the protein/modification of interest



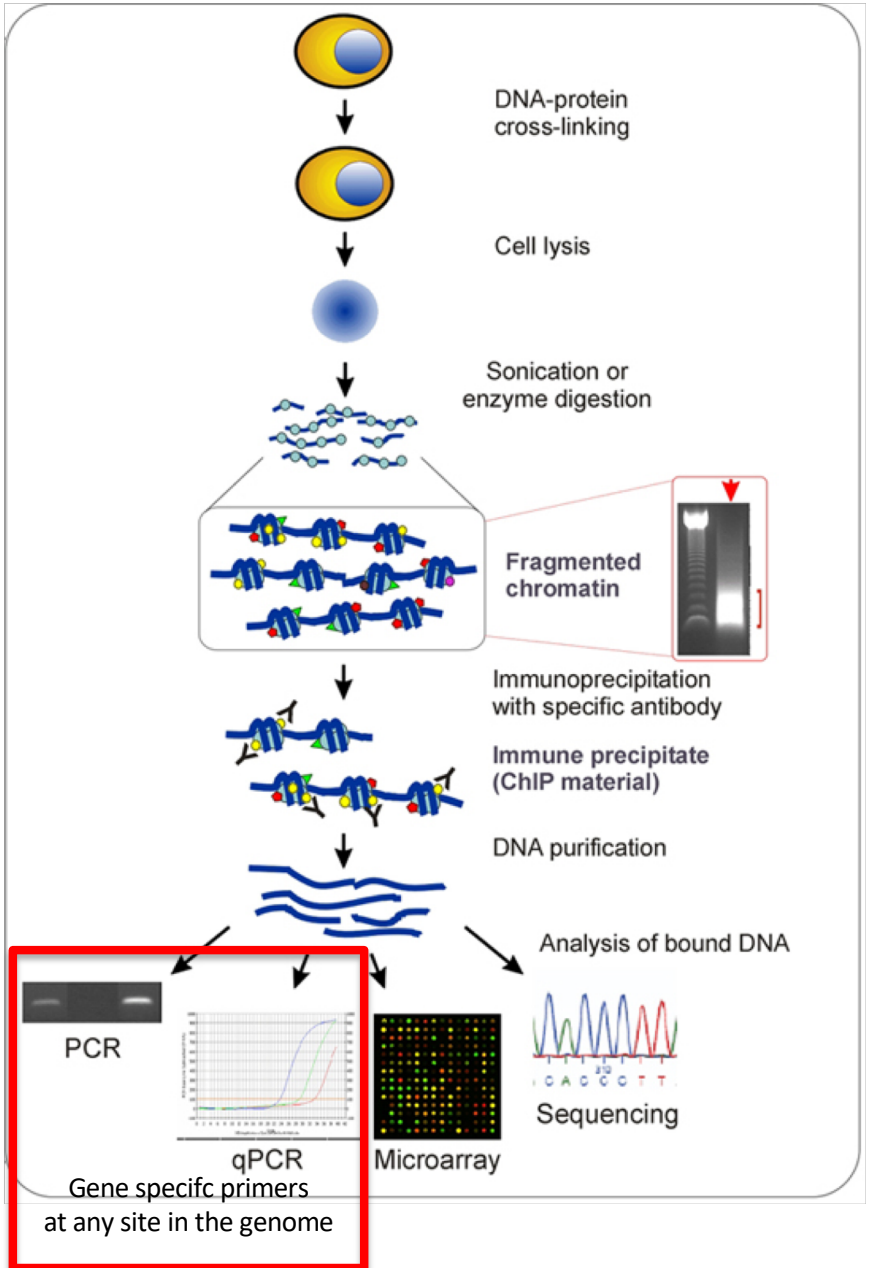
DNA from chromatin enriched in H3K9me
→ Correspond to precise sites in the genome

Methods of ChiP analysis



Lecture 3: Hallmark discovery and analysis of histone modifications

CHROMATIN IMMUNOPRECIPITATION (ChIP) → DETAILLED ANALYSIS
→ Localization of protein at a defined region - seqeunce



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i.e. Wild-type or Suv39 dn cells

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8. Measure the amount of immunoprecipitated DNA In control versus Suv39h dn cells

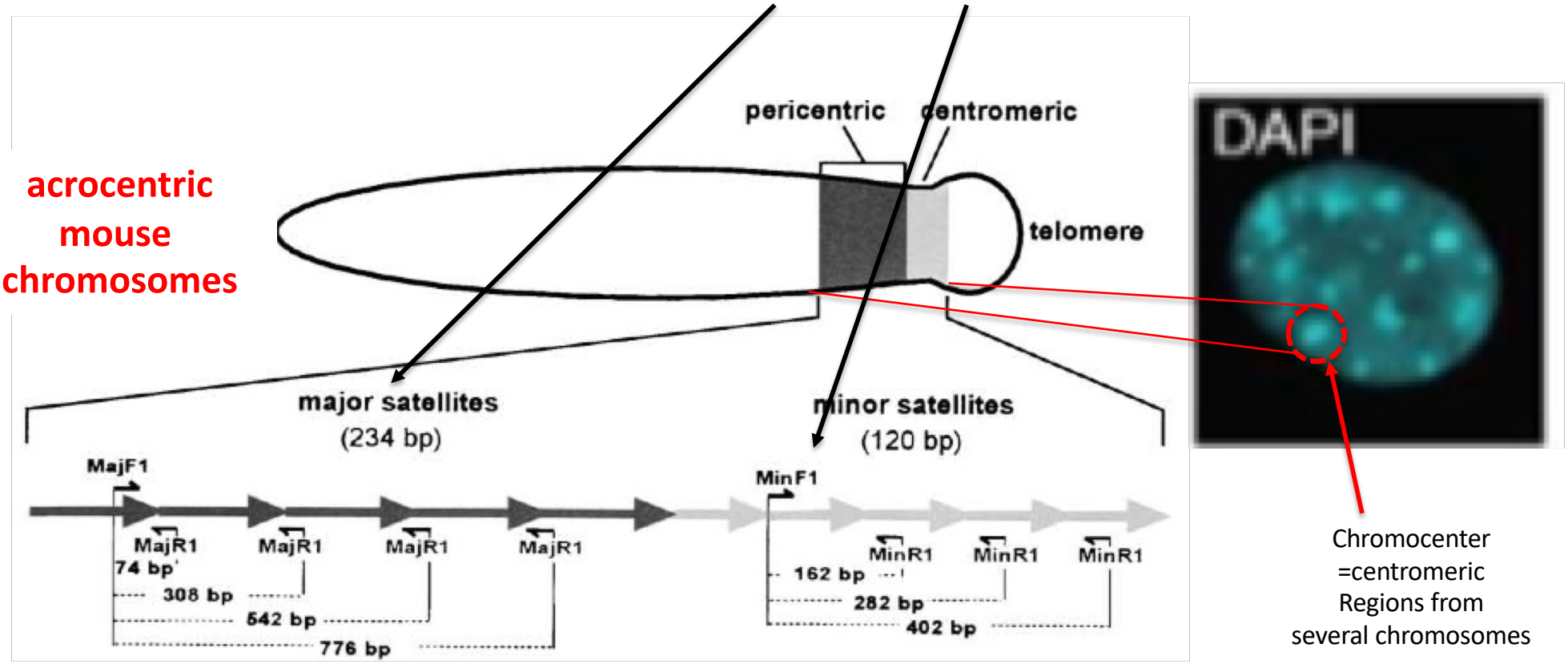
METHODS:

Quantitative PCR: design PCR oligos to amplify defined sequences in immunoprecipitates. Per PCR reaction only one Locus can be examined by real-time PCR

What are the target sites for Suv39h1 and H3K9me???

CHROMATIN IMMUNOPRECIPITATION FOLLOWED BY QUANTITTIVE PCR

Design PCR oligos that amplify major and minor satellite repeats

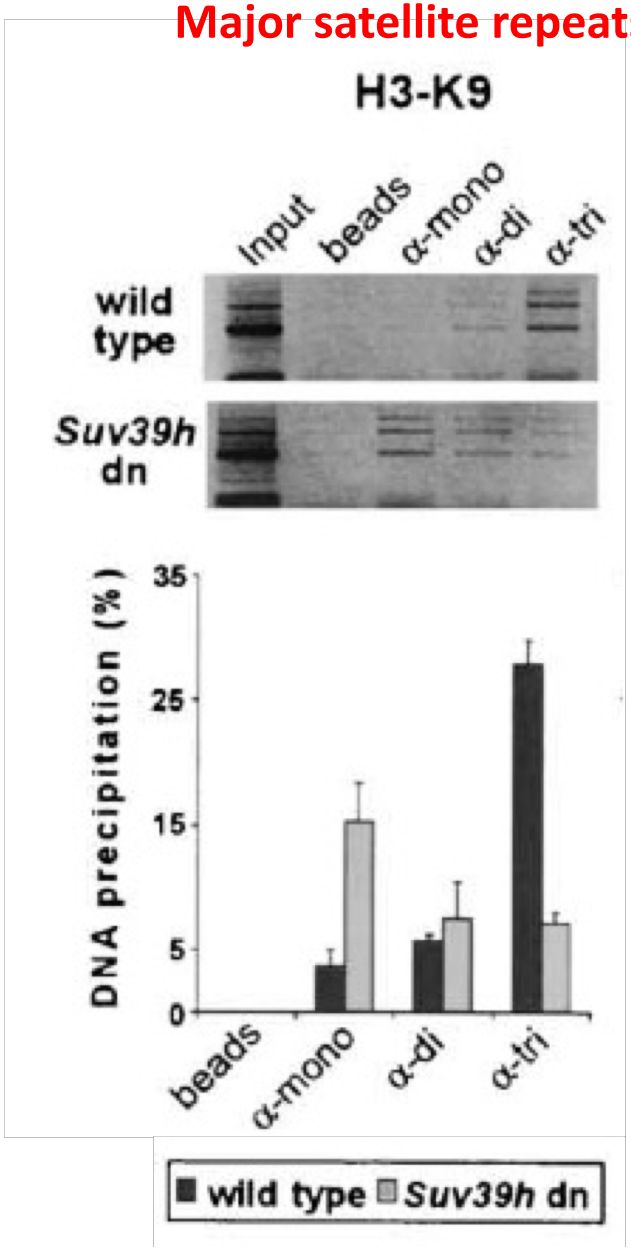


EXAMPLE: Pericentric heterochromatin in mouse cells

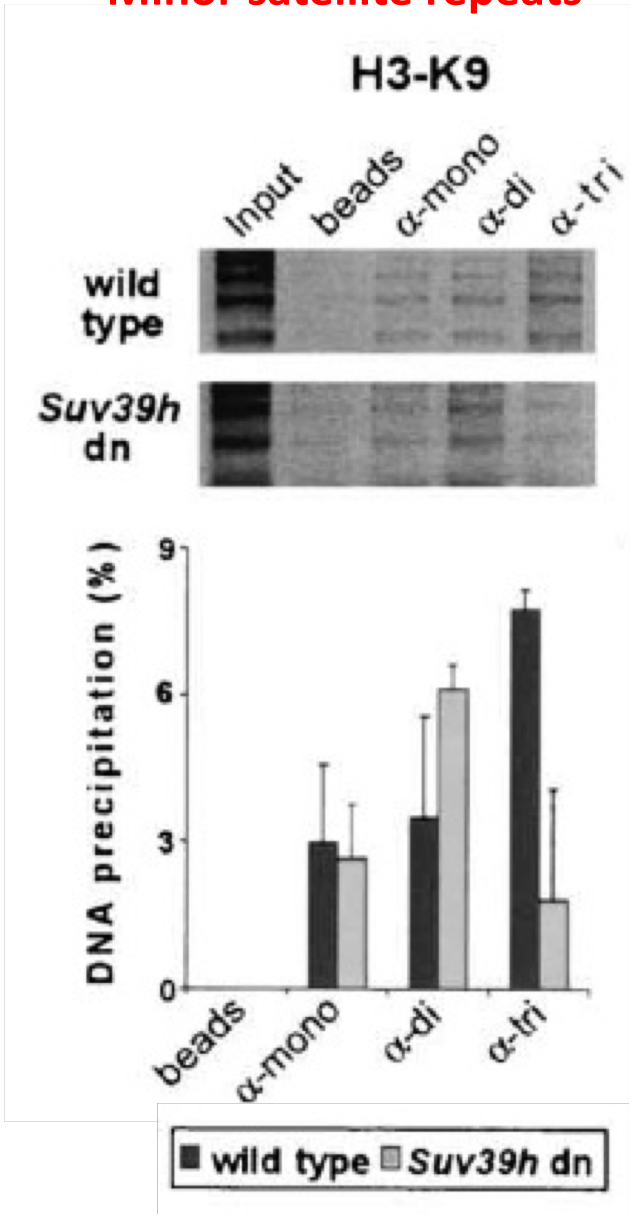
Min/Maj F1…: forward primer in unique region
Rev primer bind in every repeat

CHROMATIN IMMUNOPRECIPITATION (ChIP) COUPLED WITH PCR
→ H3K9me3 is enriched at pericentric (major+minor) repeats in mouse cells

Major satellite repeats



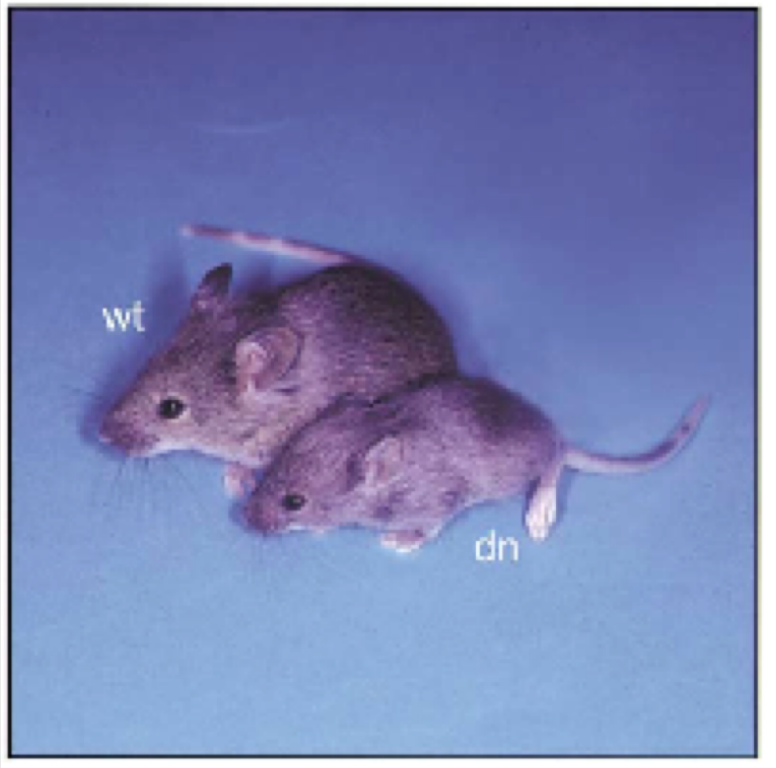
Minor satellite repeats



PCR amplification of major/minor satellite Repeats after ChiP using Antibodies that are specific for H3K9me1; H3K9me3; H3K9me3

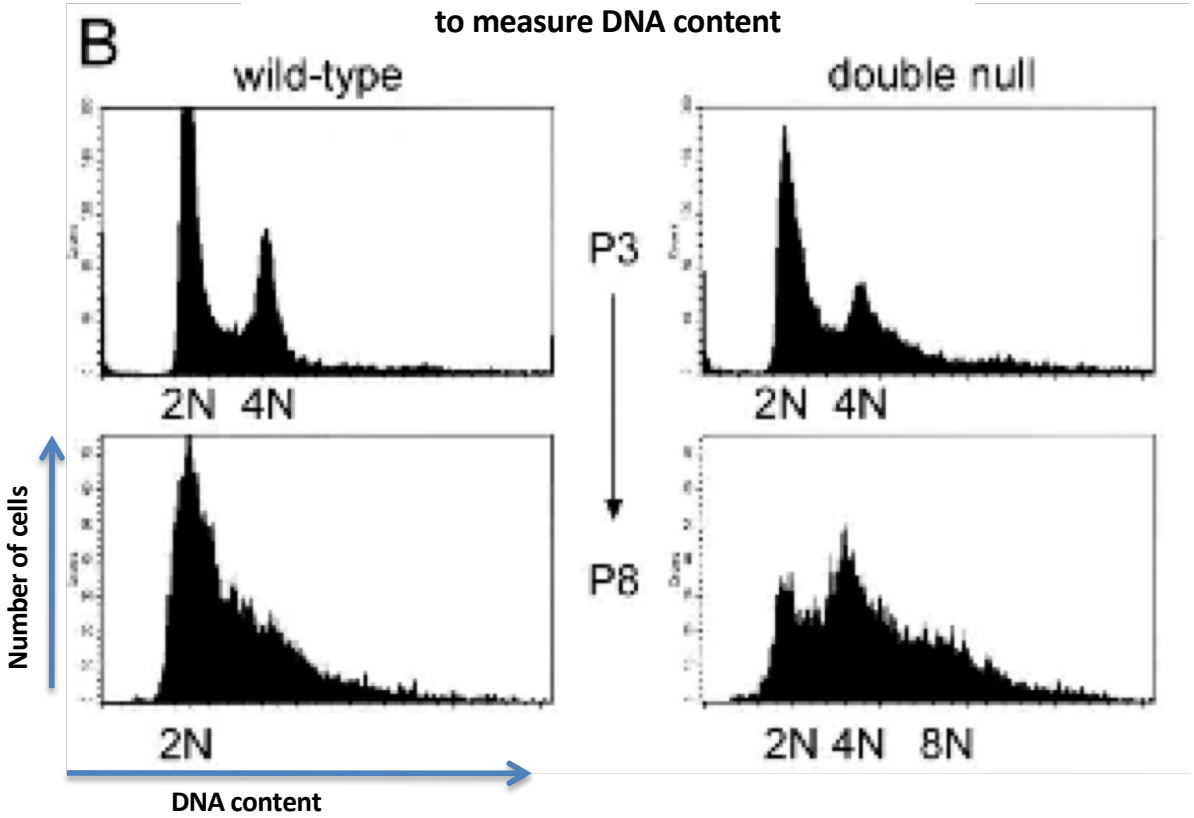
Suv39h1 is required for imposition of H3K9me3 at pericentric repeats

Lack of SUV39h HMTase activity results in genomic instability



A knock-out model system
for Suv39h1 and Suv39h2
- Loss of Suv39h1/2: smaller body size

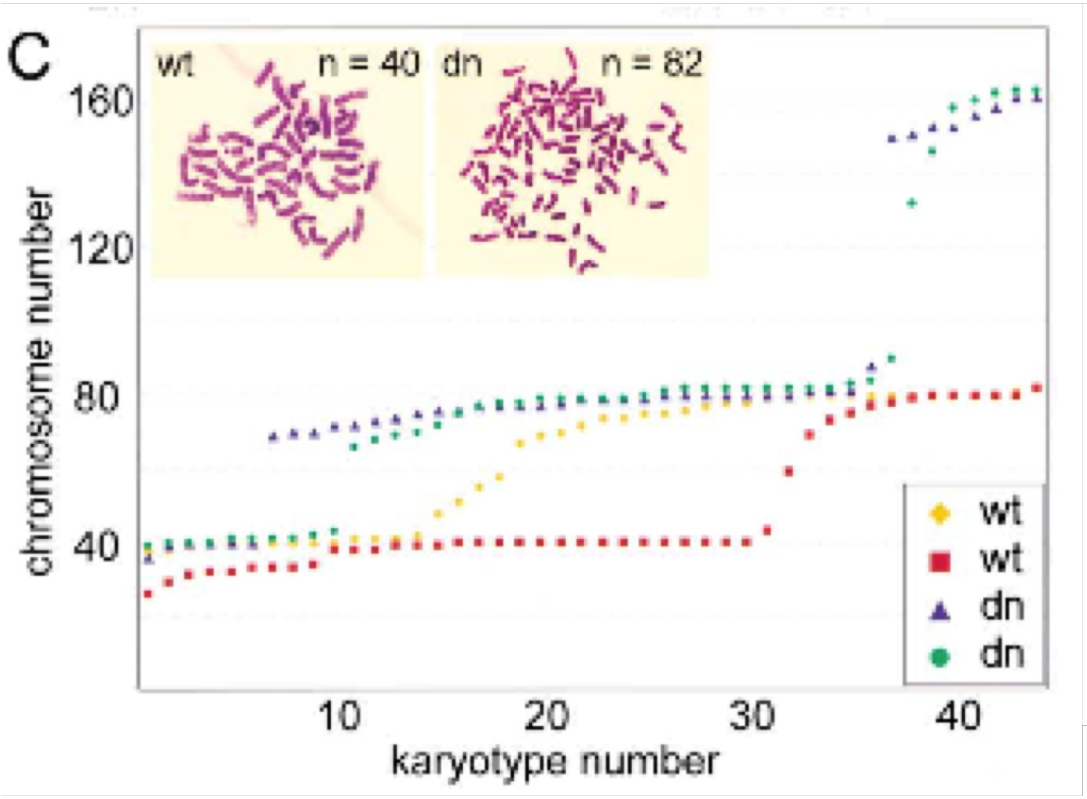
**Fluorescence activated cell sorting (FACS)
to measure DNA content**



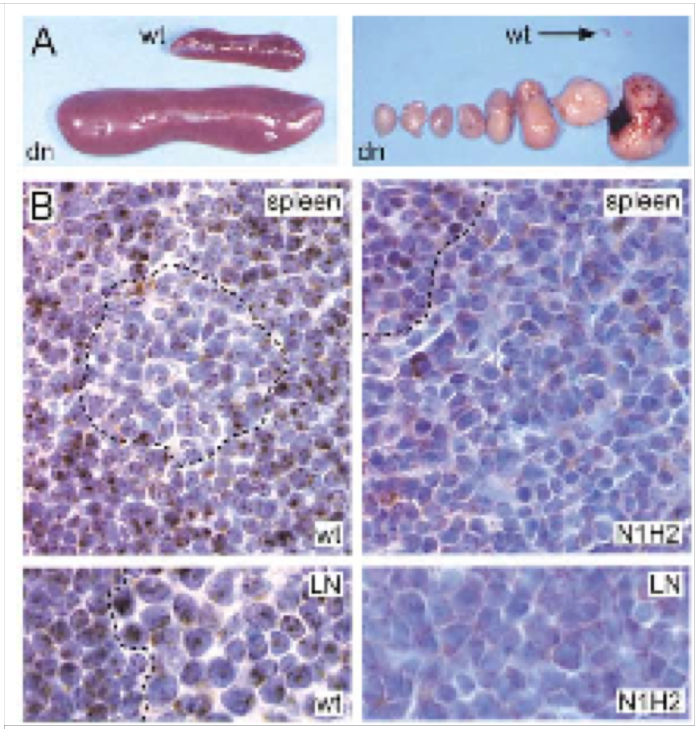
Fibroblasts from Suv39h1/2 null mice
are aneuploidy

Lack of SUV39h HMTase activity results in genomic instability

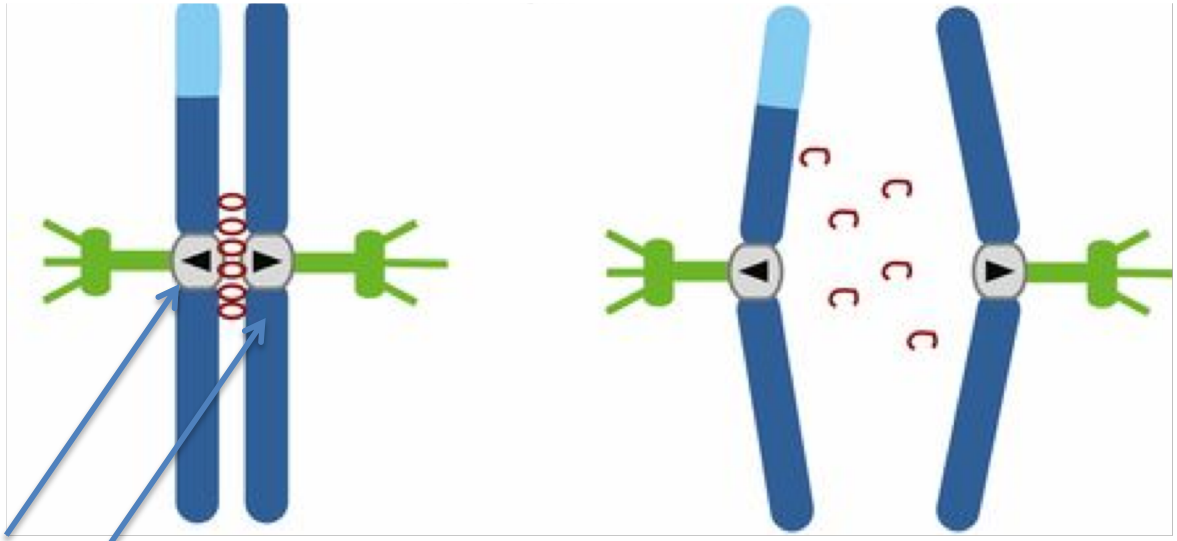
Loss of Suv39h1/2 results in increased chromosome numbers



Genomic instability in Suv39h1/2 mice increases lymphomas

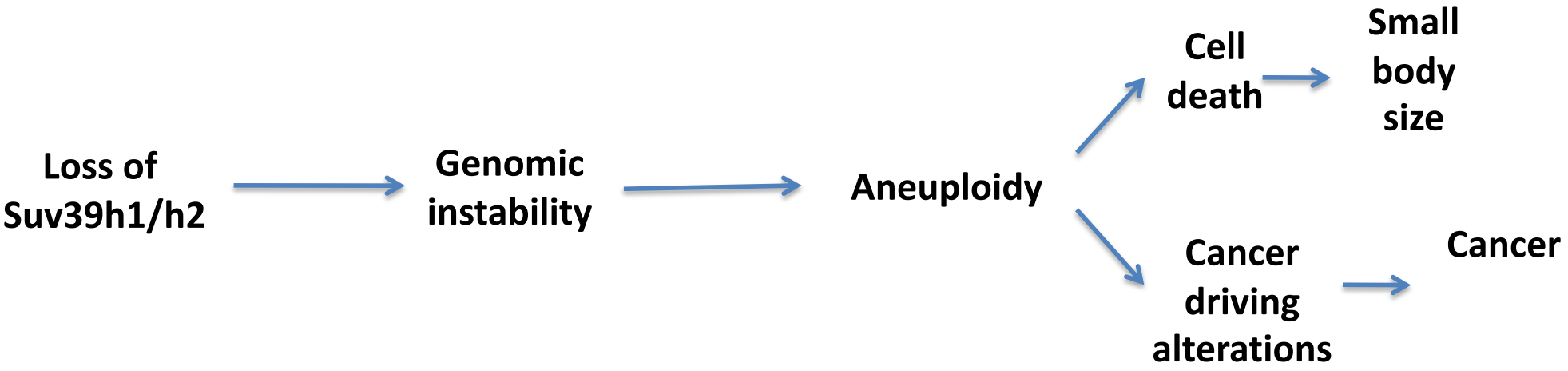


SUV39h HMTase activity is essential for fidelity in mitosis/meiosis



perientric heterochomatin

Correct heterochromatin structure supports chromosome segregation by the mitotic/meiotic spindle apparatus



Lecture 3: Hallmark discovery and analysis of histone modifications

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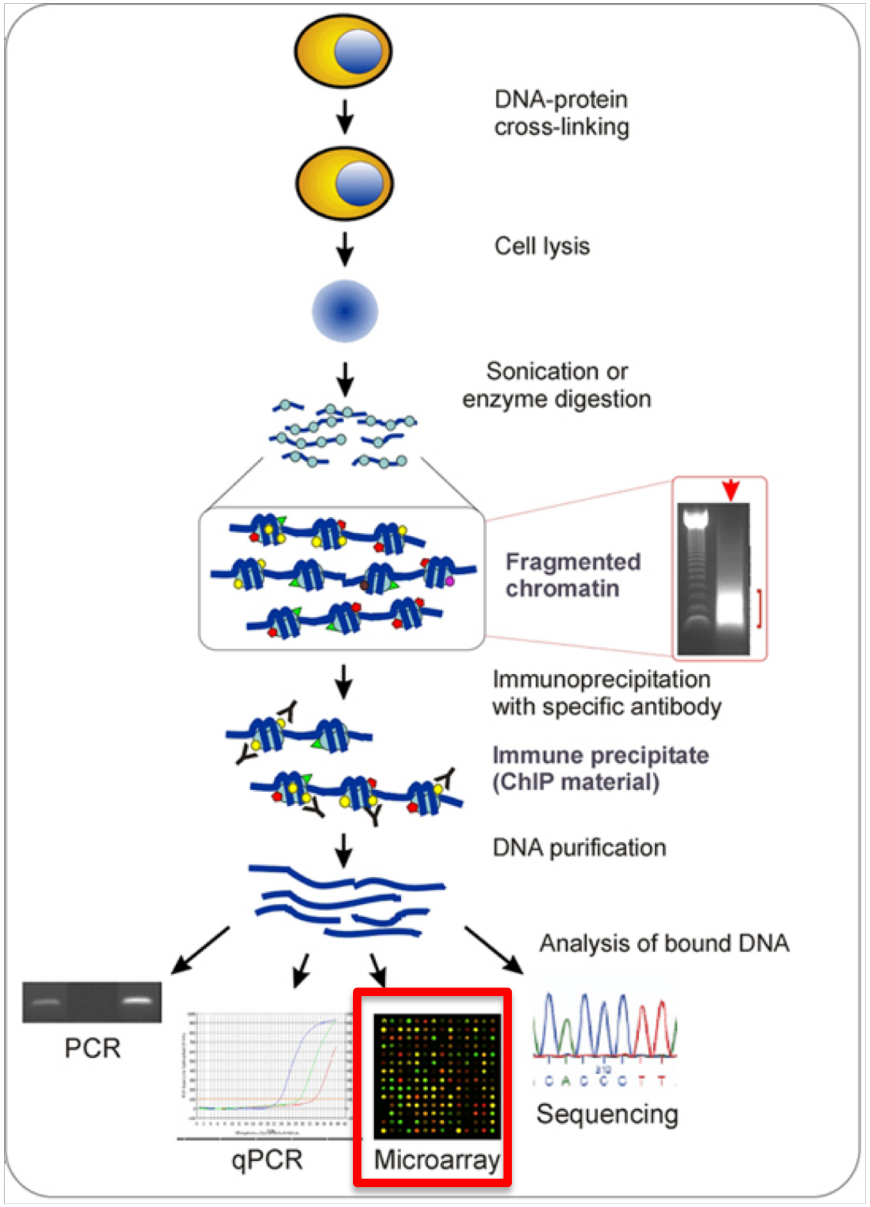

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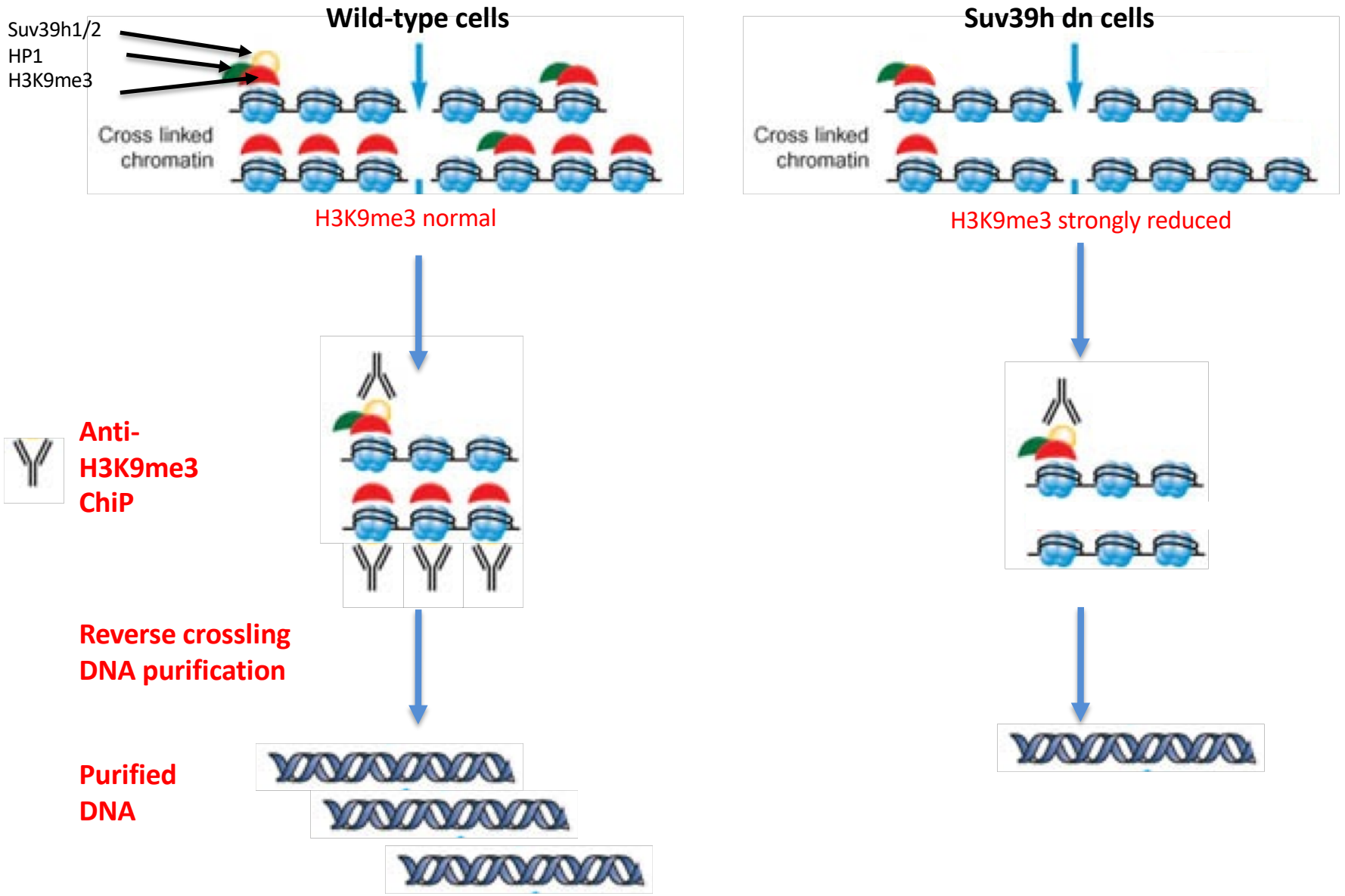
ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

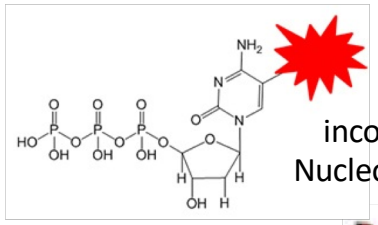


ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

Purified DNA From ChIP

Wild-type cells

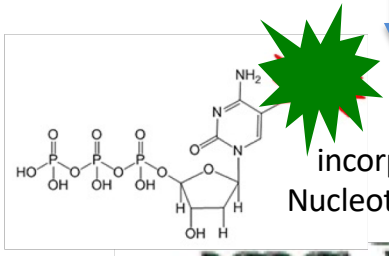
Suv39h dn cells



Fluorescently labeled probe

Denaturalization of DNA;
priming of DNA synthesis;
incorporation of fluorescently labeled
Nucleotides into newly synthesized strand

Cy5- labeled
(~650 excitation, 670 nm emission)



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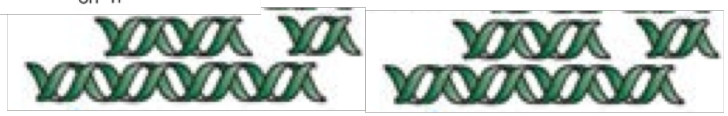
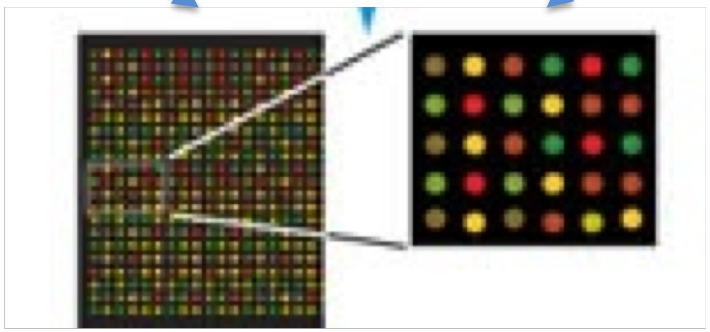


Cy3- labeled
(550 nm excitation, ~570 nm emission)

Mix probes

Hybridize to a genome array



Measure Green/Red ratio

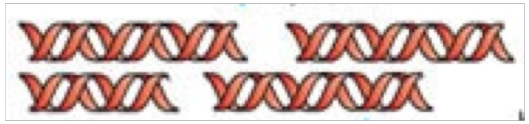
ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

Purified DNA From ChIP

Wild-type cells

Suv39h dn cells

Fluorescently labeled probe



Cy5- labeled
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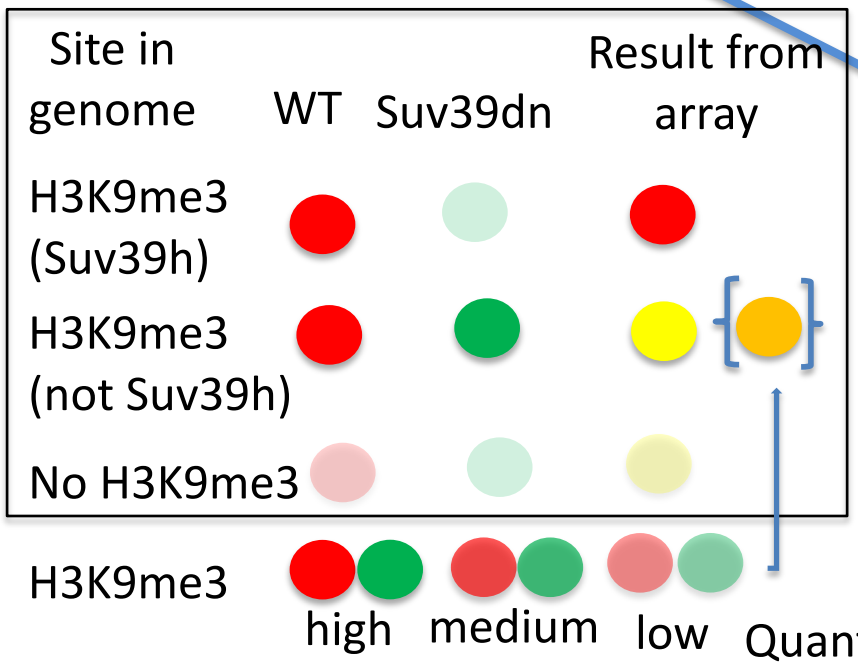
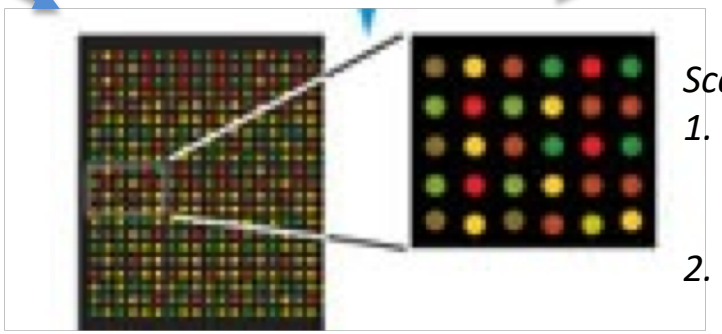
Cy3- labeled
(550 nm excitation, ~570 nm emission)



Mix probes



Measure Green/Red ratio
Hybridize to a genome array

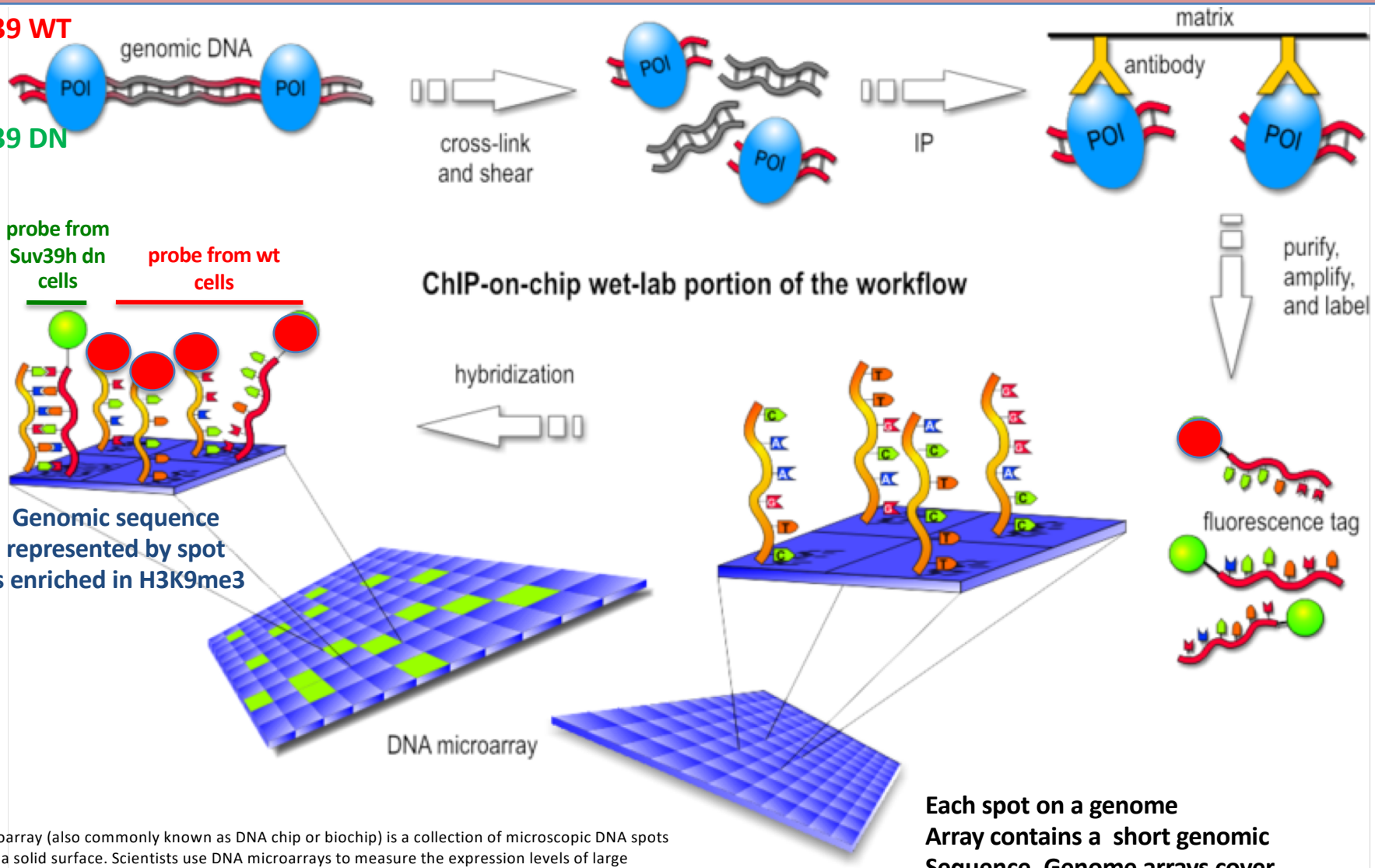
Scan:

1. *Red channel (+normalize signal)*
2. *Green channel (+normalize signal)*

ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome

1. SUV39 WT

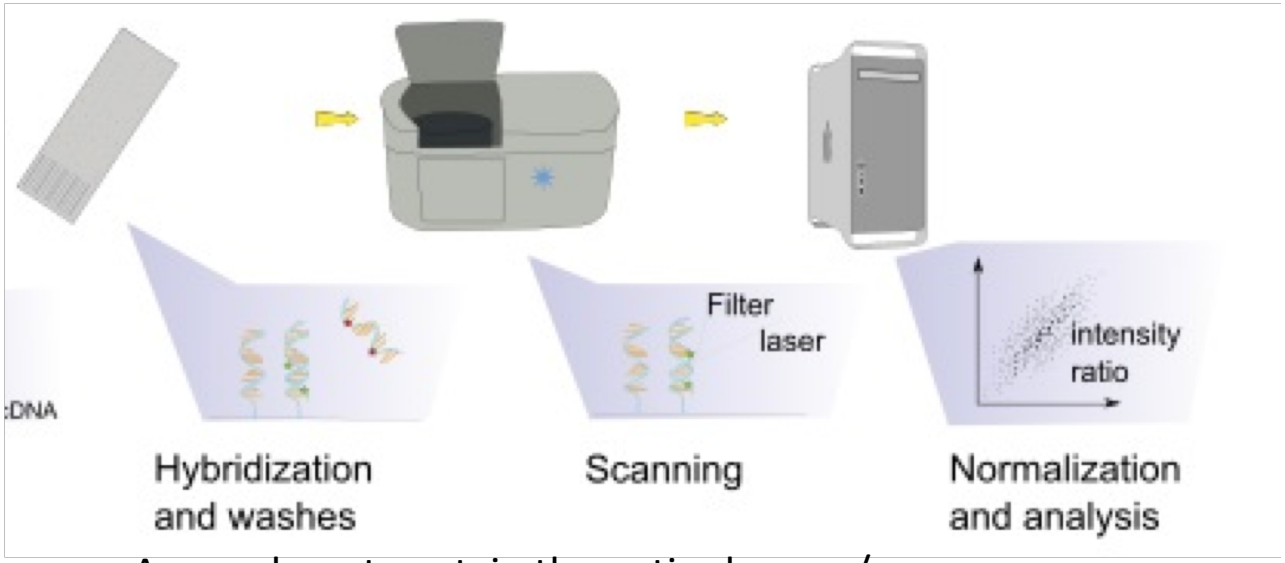
2. SUV39 DN

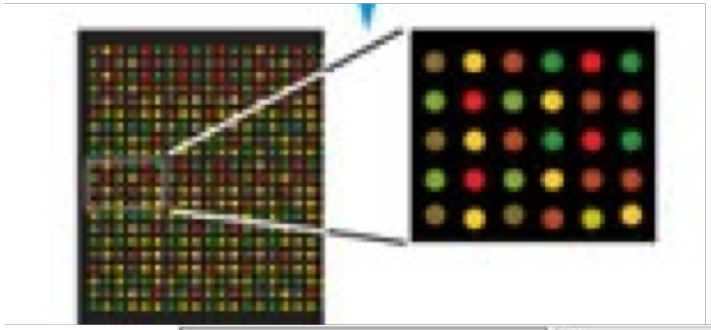


A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10−12 moles) of a specific DNA sequence, known as probes (or reporters or oligos). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target

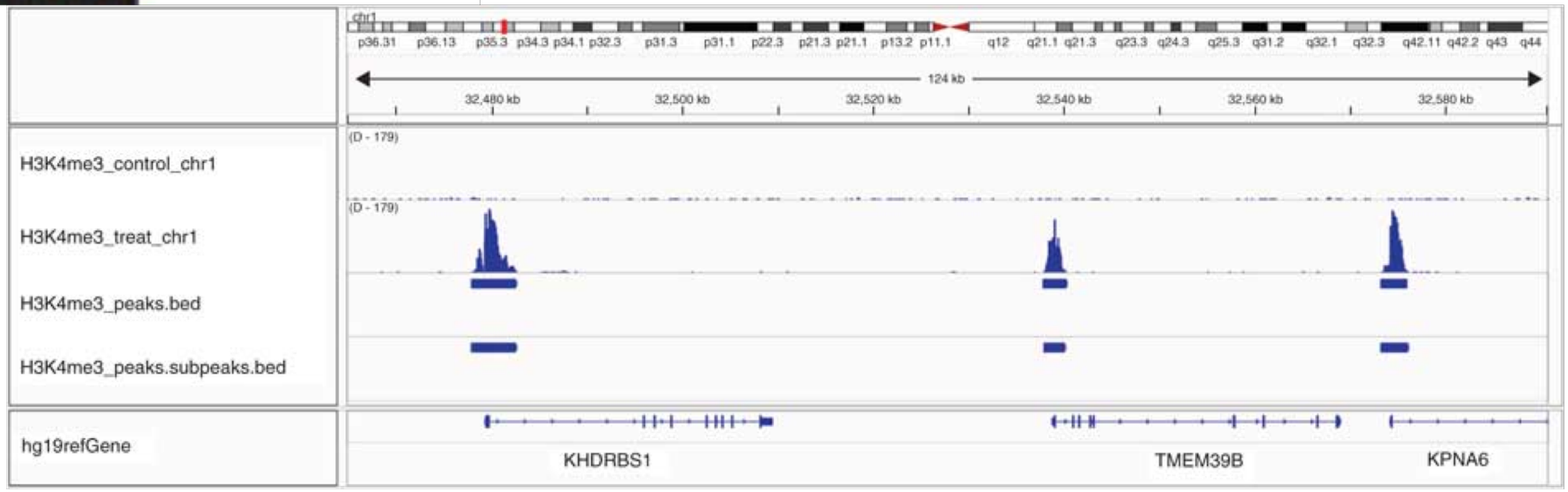
Each spot on a genome Array contains a short genomic Sequence. Genome arrays cover a large part of the Genome
A ChIP holds ca 20000 spots

ChIP on ChIP: Analysis of epigenetic information across a high number of genomic sites in the genome





Arrays do not contain the entire human/mouse genome
Are enriched for particular seqeunces according to need
(promoter, enhancer, etc…)



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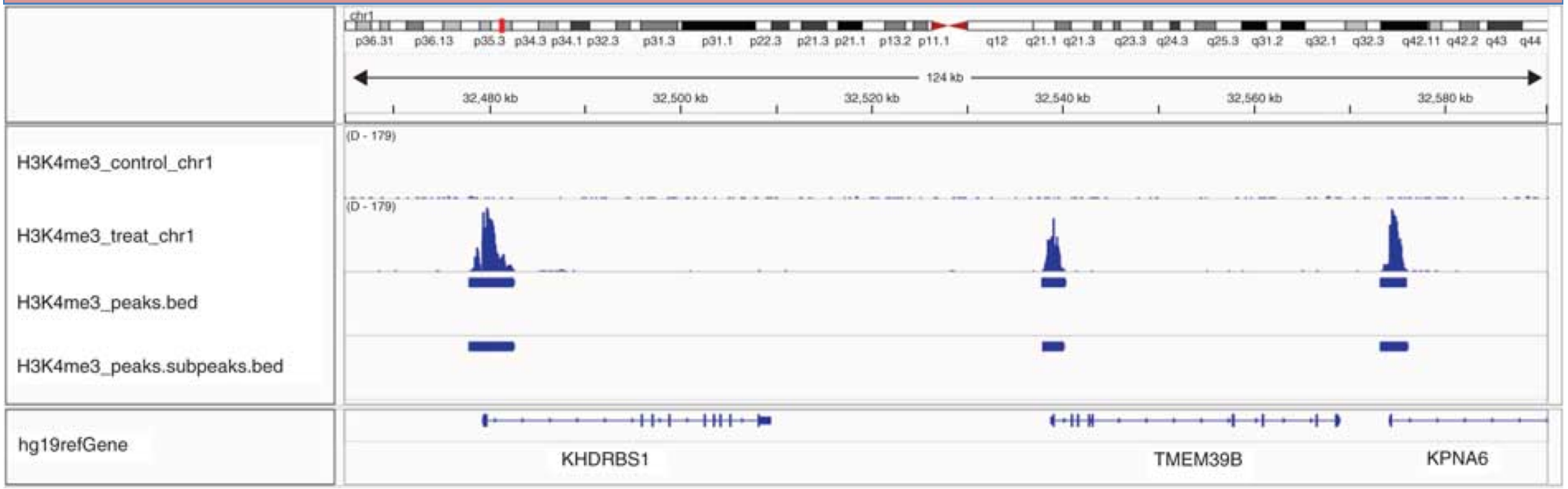


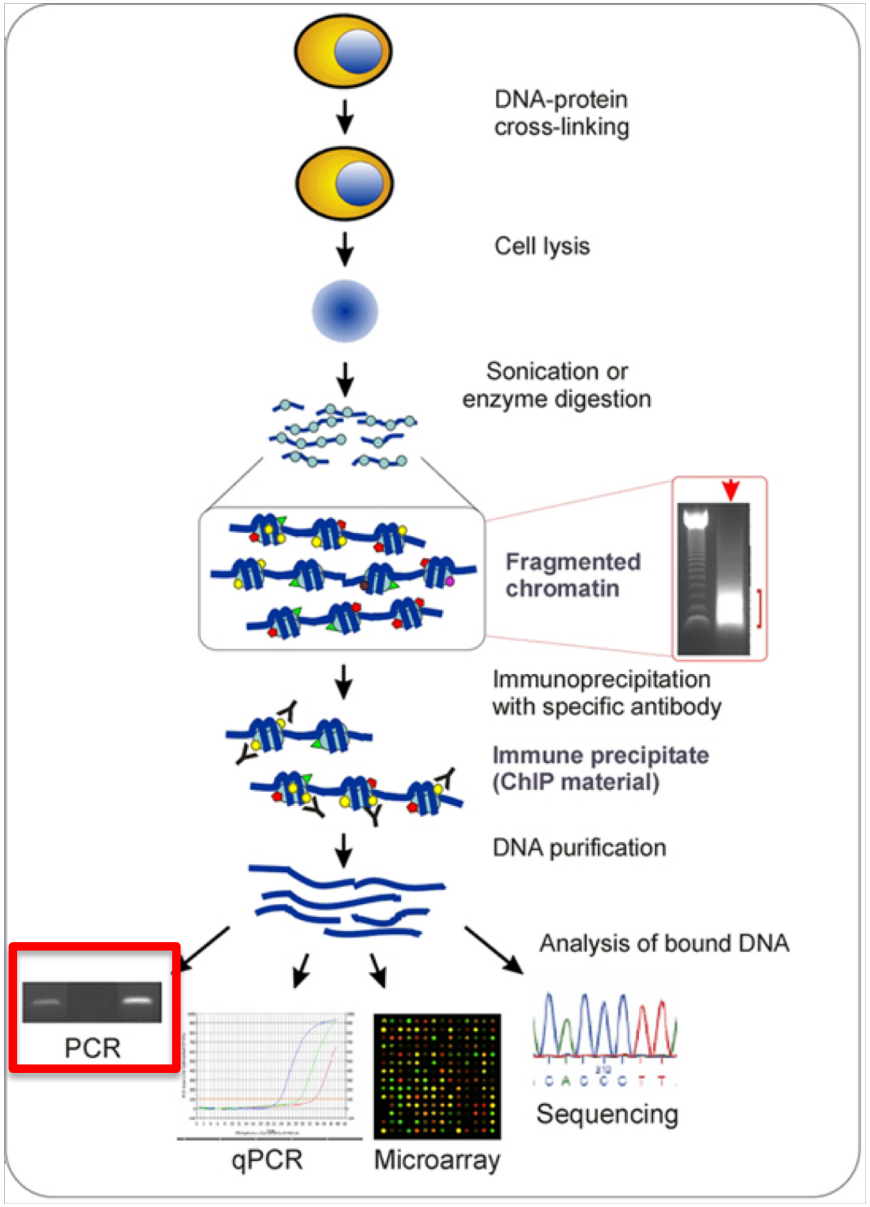
Advantage: low tech, cheap

Disadvantage: low resolution, no data on number of molecules – just proportions; laborious to reach a good genome coverage

Already outdated → state of the art: ChIP seq

Lecture 3: Hallmark discovery and analysis of histone modifications

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERATION OF GENOME WIDE EPIGENTIC MAPS



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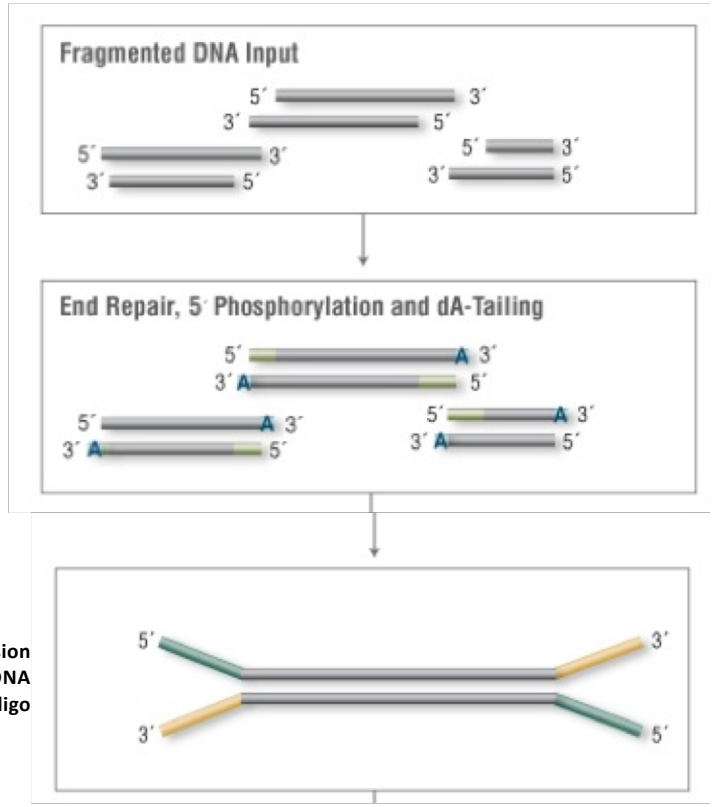
METHODS:

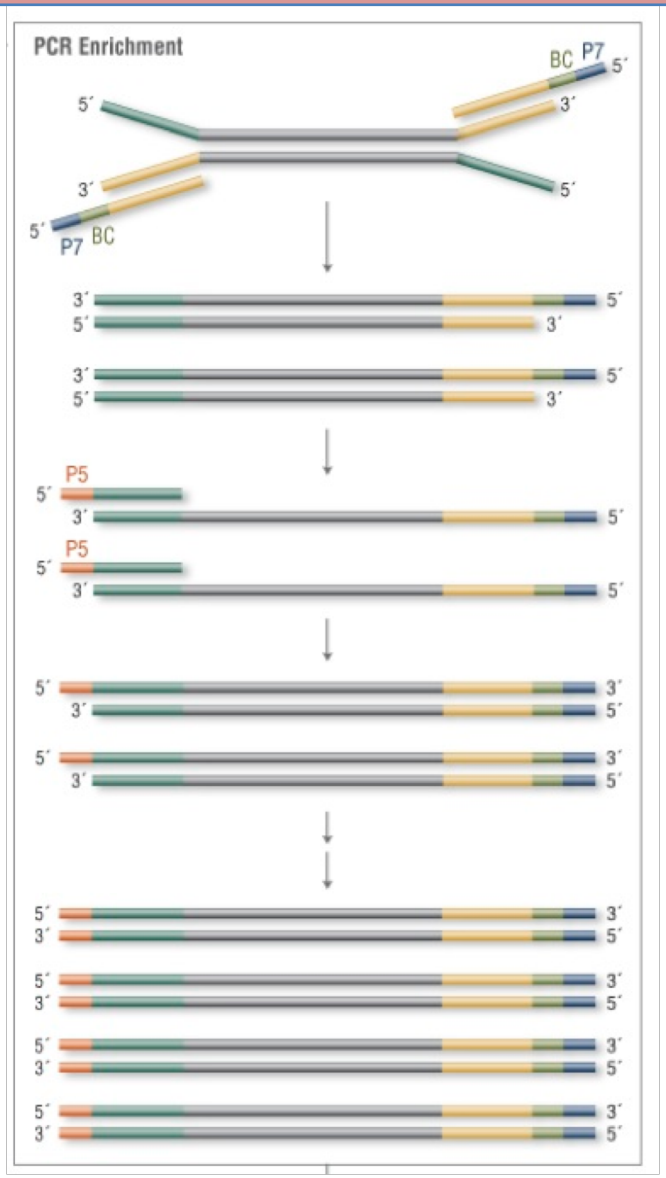
Massive parallel sequencing of immunoprecipitated DNA
Permits to obtain epigenetic information on the single nucleotide level

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERASTION OF GENOME WIDE EPIGENTIC MAPS

Use immunoprecipitated DNA to generate DNA libraries:



fusion of DNA with linker oligo



Linkers serve as uniform primer binding sites. This allows the amplification of the entire DNA library using only 2 types of oligonucleotides

Amplified library

READY FOR MASSIVE PARALLEL SEQEUNCING

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERASTION OF GENOME WIDE EPIGENTIC MAPS

Illumina Massively Parallel Sequencing

https://www.illumina.com/company/video-hub/pfZp5Vgsbw0.html

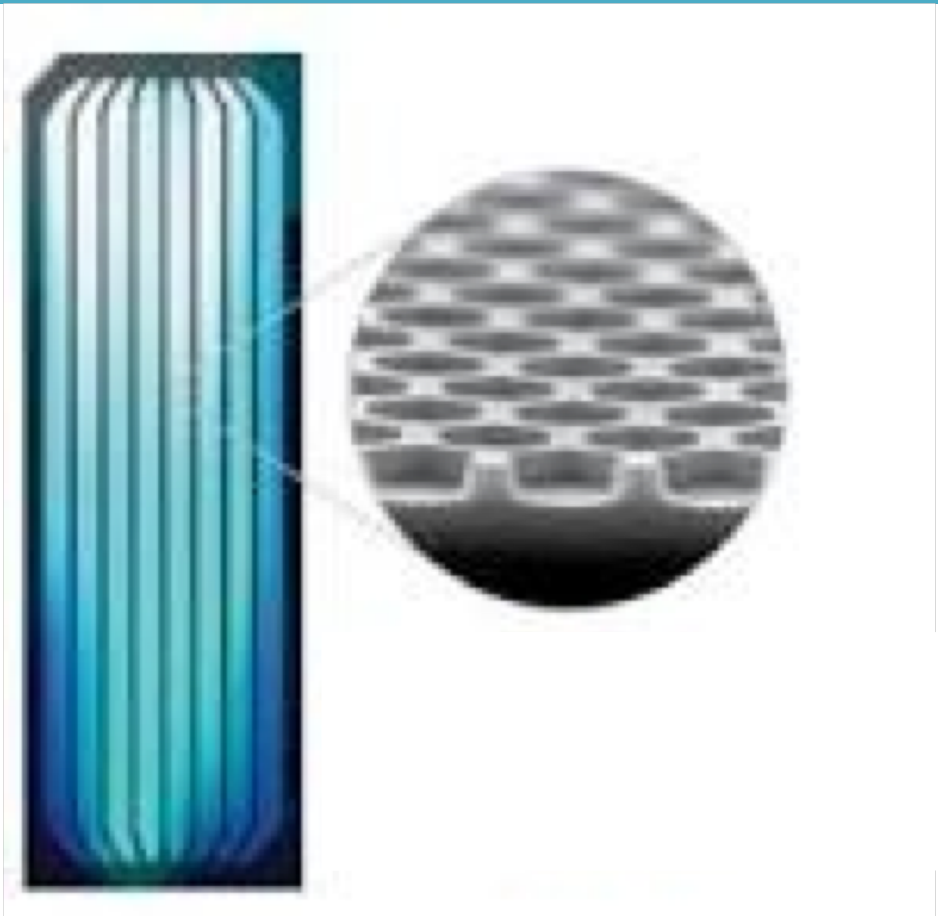
The heart of the Illumina Massive Parallel Sequencer is the "FLOW-CELL". A surface with millions of small wells that allow thousands of Sanger-sequencing reaction In parallel = "massive parallel sequencing". In each well a SINGLE MOLECULE of DNA Is amplified and sequenced

Illumina offers the most potent massive sequencing instruments – leader on the market

https://www.youtube.com/watch?v=pfZp5Vgsbw0

ChIP seq: Analysis of epigenetic information on the single nucleotide level
→ GENERASTION OF GENOME WIDE EPIGENTIC MAPS

CLUSTER AMPLIFICATION:



Flow cell contains surface with millions of wells

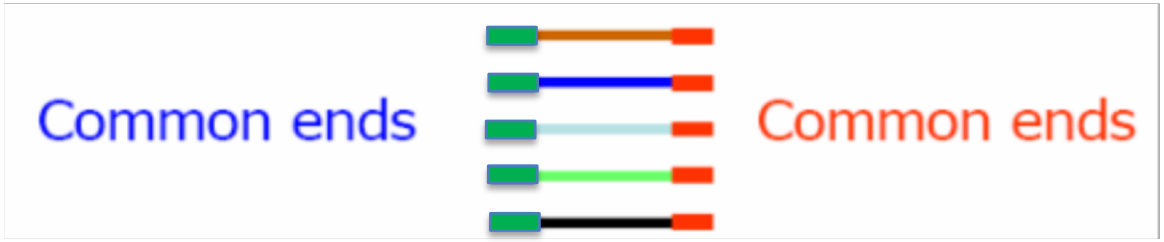
→Each well contains beads mounted with 2 species of oligonucleotides that hybridize with adaptor oligos of DNA library

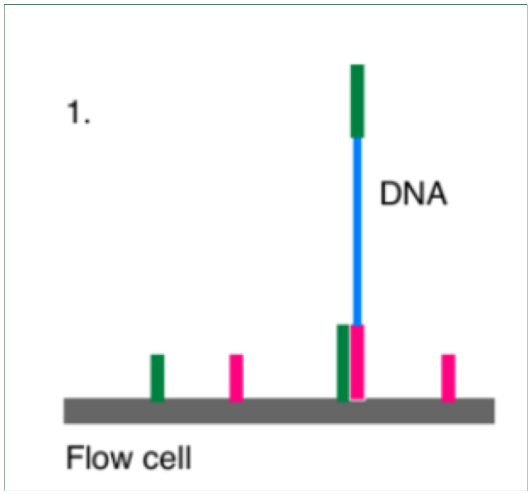
→DNA library will be loaded onto the flow cell in a determined concentration:

ONLY ONE MOLECULE OF DNA WILL BE PROCESSED FOR SEQUENCING IN A SINGLE WELL

CLUSTER AMPLIFICATION:

-making DNA library (~300bp fragments)
-ligation of adapters **A** and **B** to the fragments





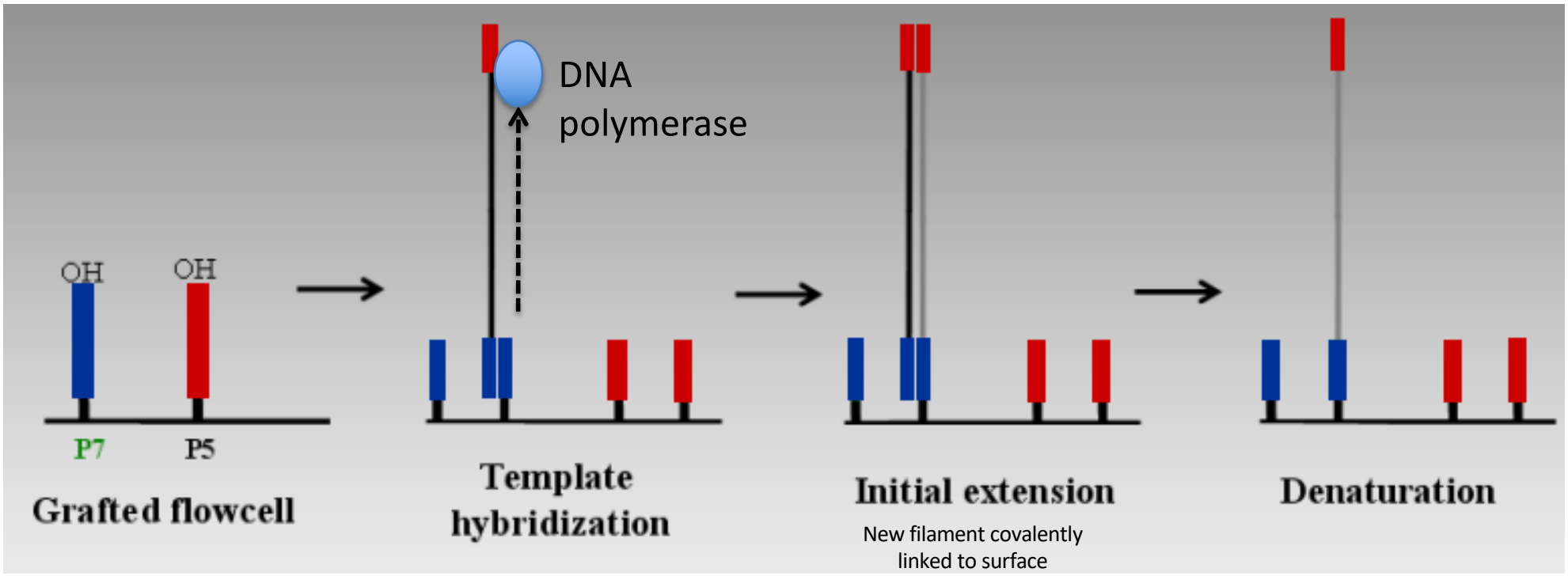
1 well, covered with
millions of 2 types of oligos

1 well in a flow-cell with
billions of wells

- ***complementary*** primers are ligated to the surface
- pairing with ChiP ed ssDNA at random position in the well of the flow cell

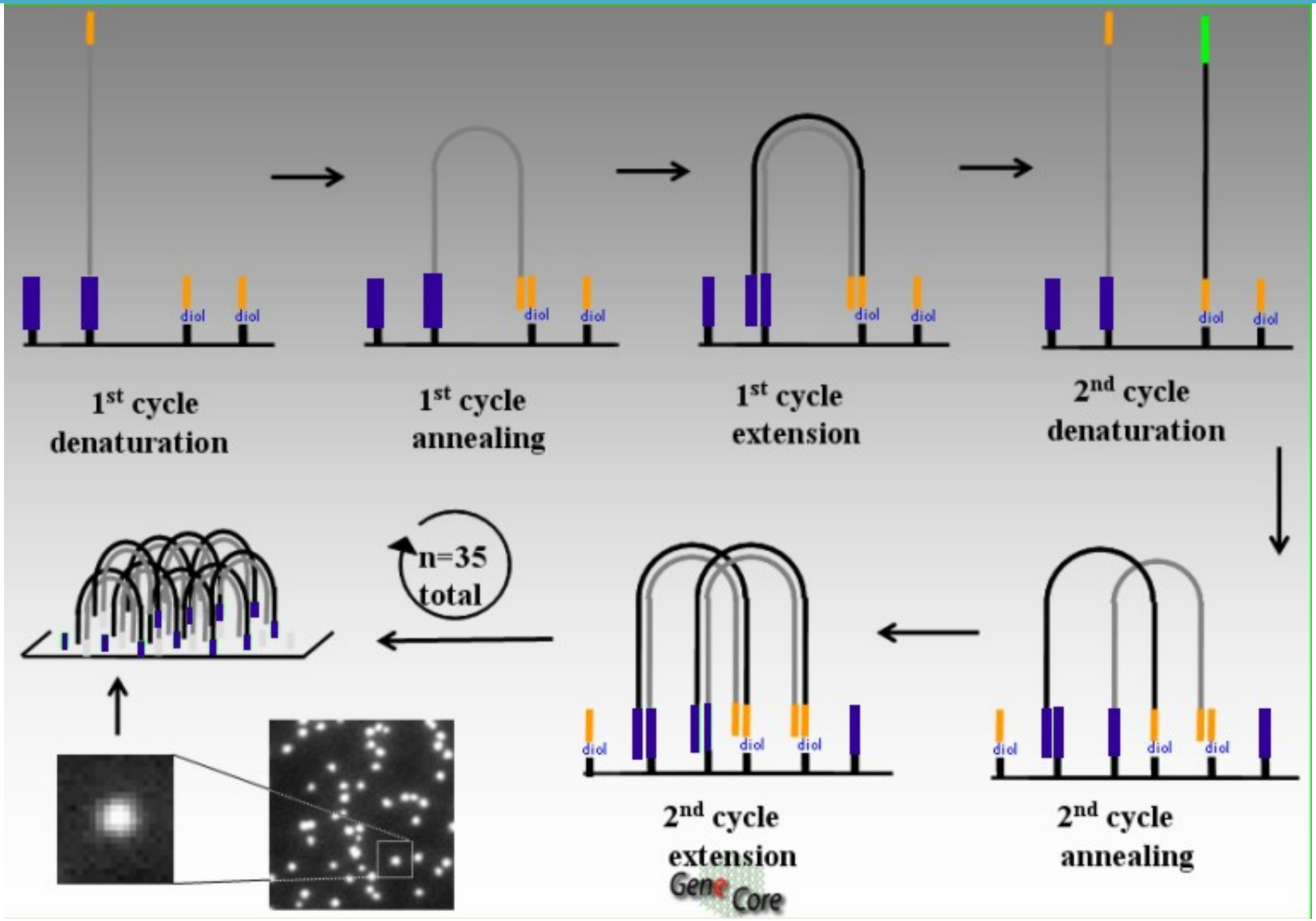
CLUSTER AMPLIFICATION:

Bridge amplification: takes place on surface of beads (each bead is mounted with 2 species of oligos; each oligo can hybridize to a DNA library fragment): initiation



On the surface: complementary oligos

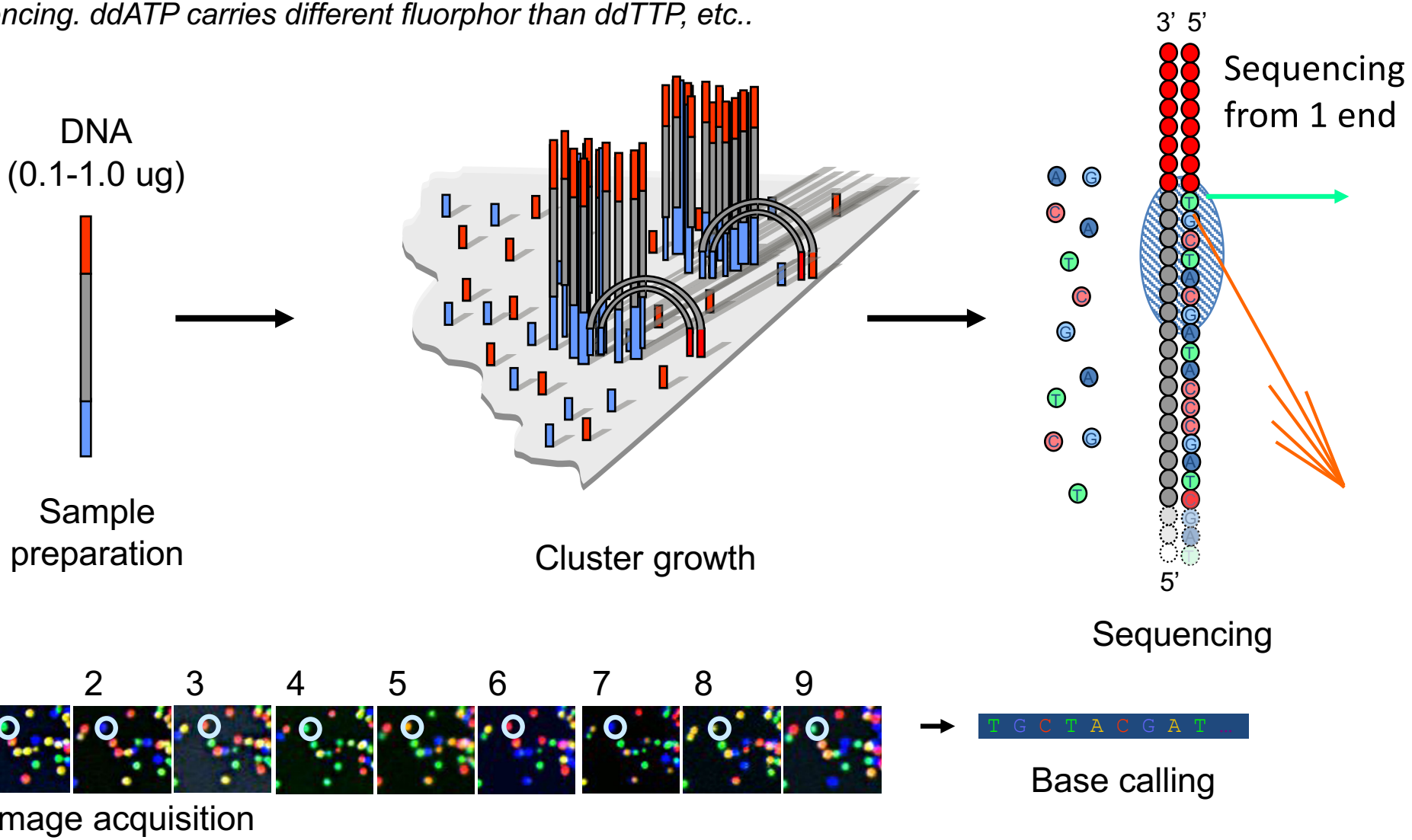
CLUSTER AMPLIFICATION:



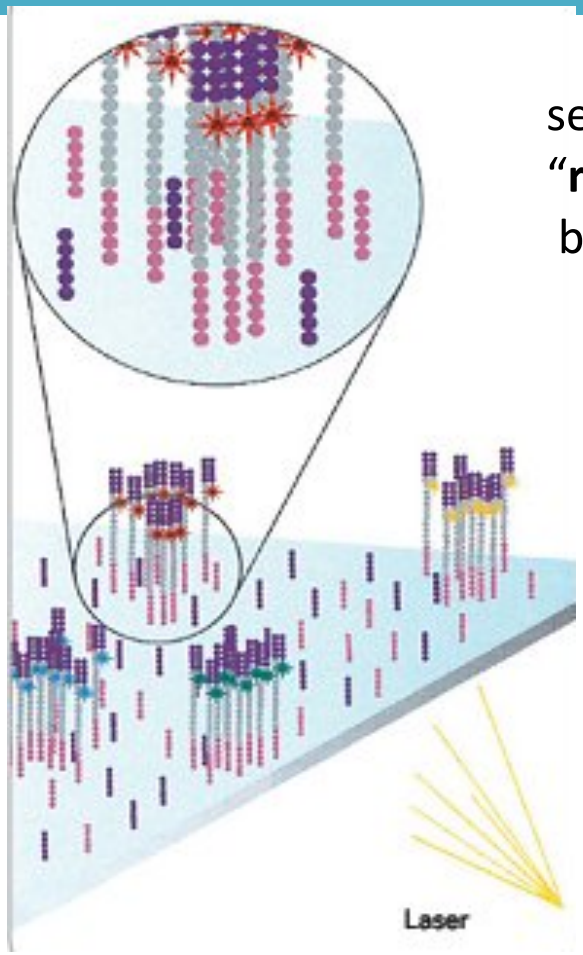
Illumina Sequencing Technology

Robust Reversible Terminator Chemistry Foundation

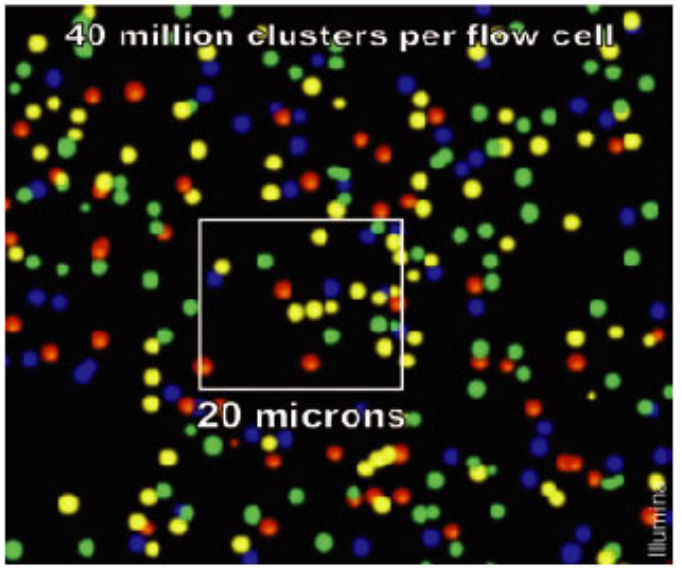
In each round of sequencing a fluorescently labelled ddNTP will be used for sequencing. ddATP carries different fluorphor than ddTTP, etc..



Illumina: massive parallel sequencing:



sequencing by synthesis:
"**reverible terminator**" nucleotides
blocked + fluorescently labeled



1. **Synthesis = incorporation of fluorescent nucleotide: blocking synthesis**
2. **4. Scanning of fluorescent signal**
3. **dye cleavage + elimination**
4. **wash step**
1. **Synthesis = incorporation of fluorescent nucleotide: blocking synthesis**

READ LENGTH: ca: 150nt from each primer (2x150nt = 300nt)

illumina.com

Illumina: paired end sequencing increases information content

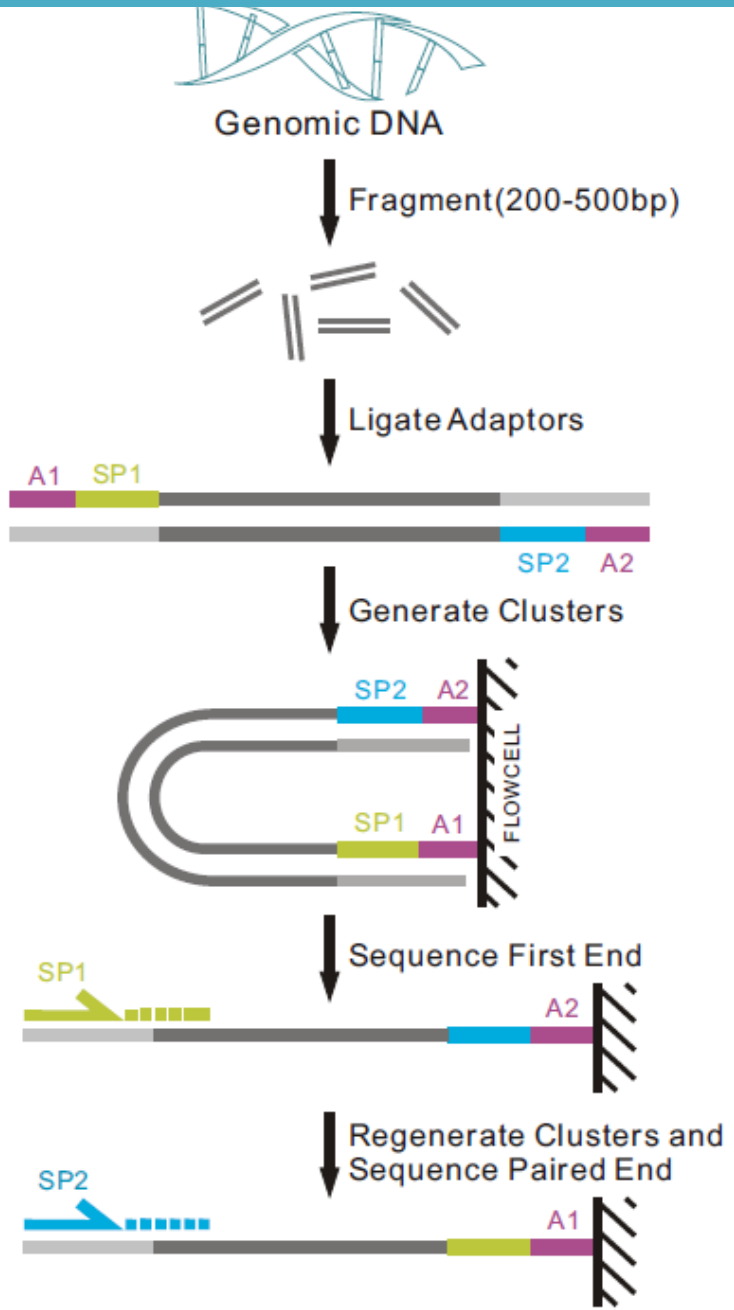


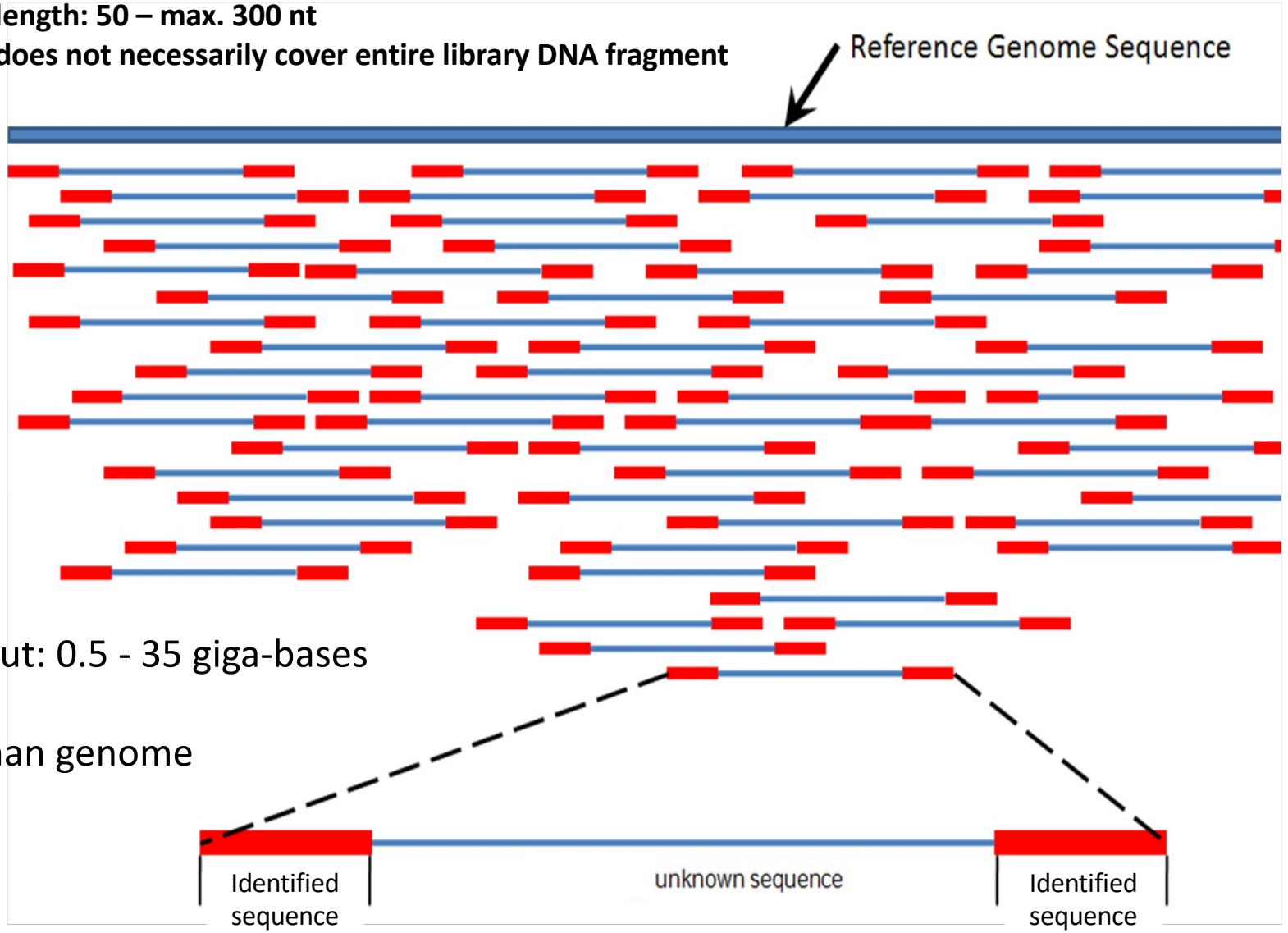
Figure 1-2-1 Pipeline of paired-end sequencing (www.illumina.com)

https://www.youtube.com/watch?v=9YxExTSwgPM

After 1° strand sequencing, A1 anneals to A1 in nanowell-->DNA synthesis --> template strand cleaved off → new strand sequenced

Data analysis: obtained sequence reads are aligned along genomic DNA sequence → high number of reads necessary to obtain full sequence coverage

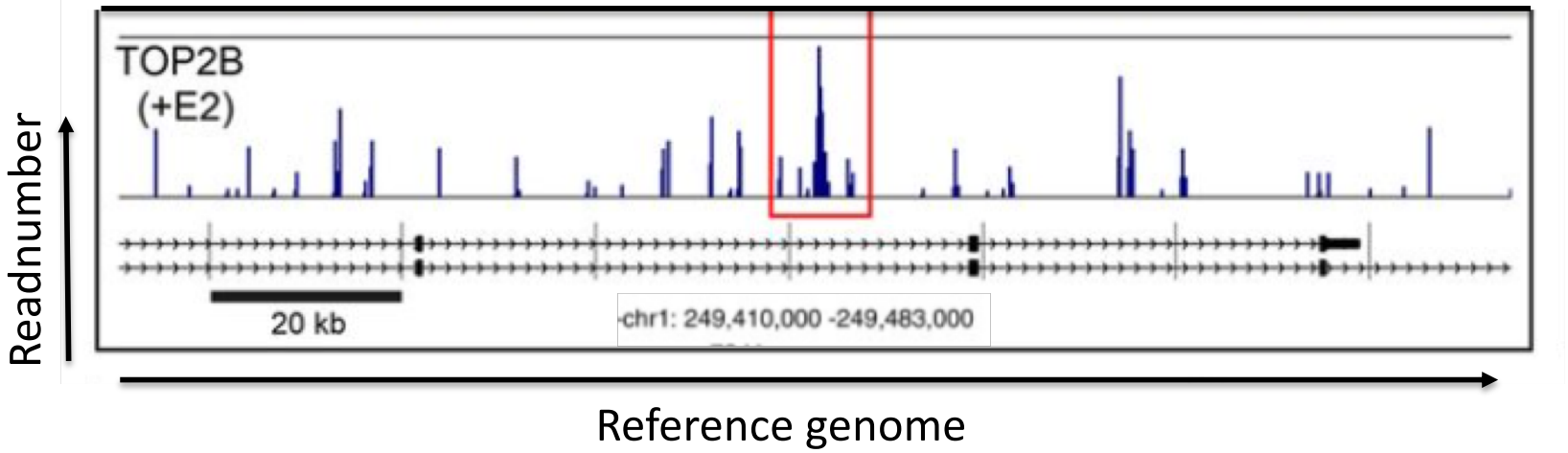
Read length: 50 – max. 300 nt
Read does not necessarily cover entire library DNA fragment



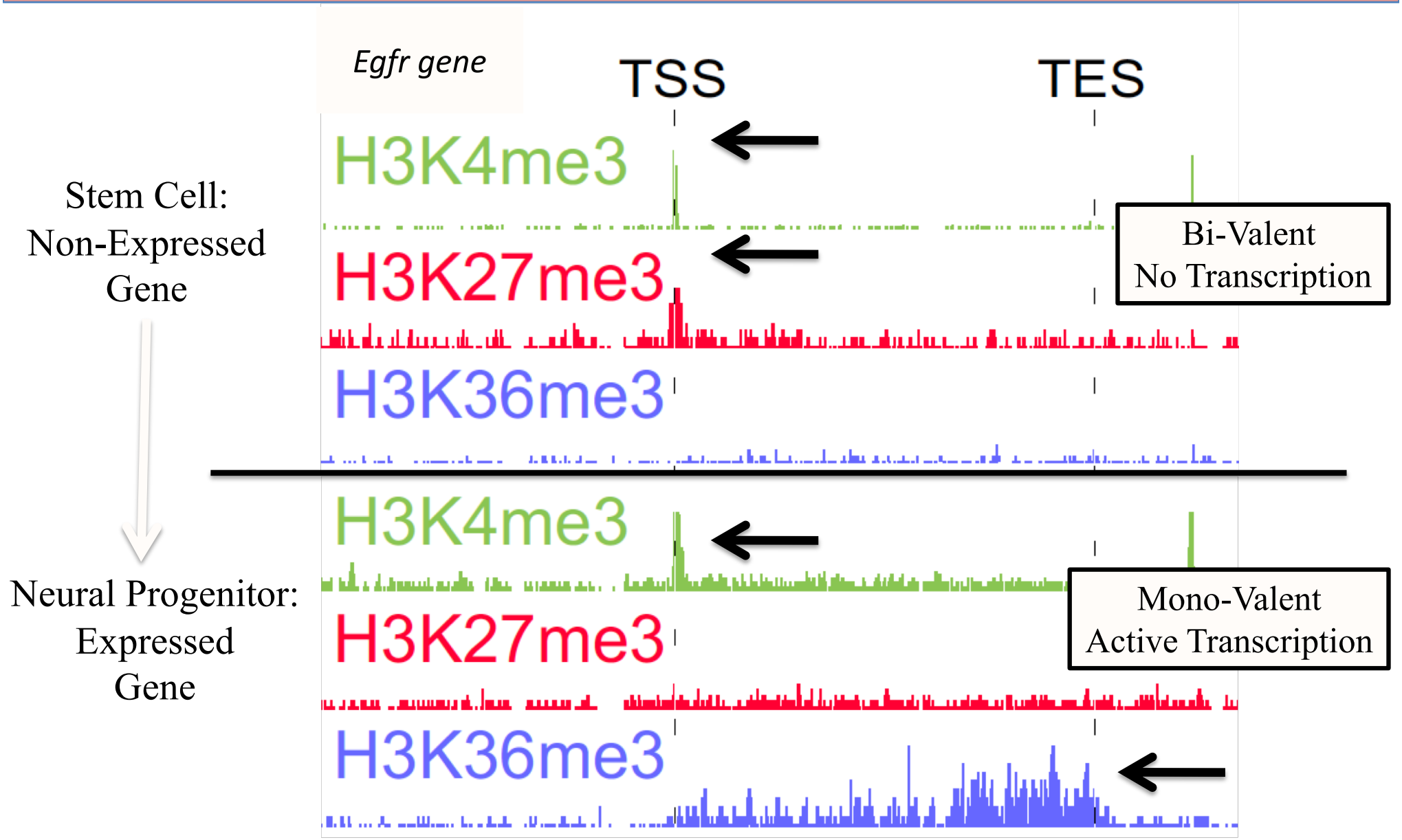
Max. output: 0.5 - 35 giga-bases
$=3.5*10^{10}$
= 10x human genome

Sequence derived from one amplified cluster

Data analysis: obtained sequence reads are aligned along genomic DNA sequence → high number of reads necessary to obtain full sequence coverage

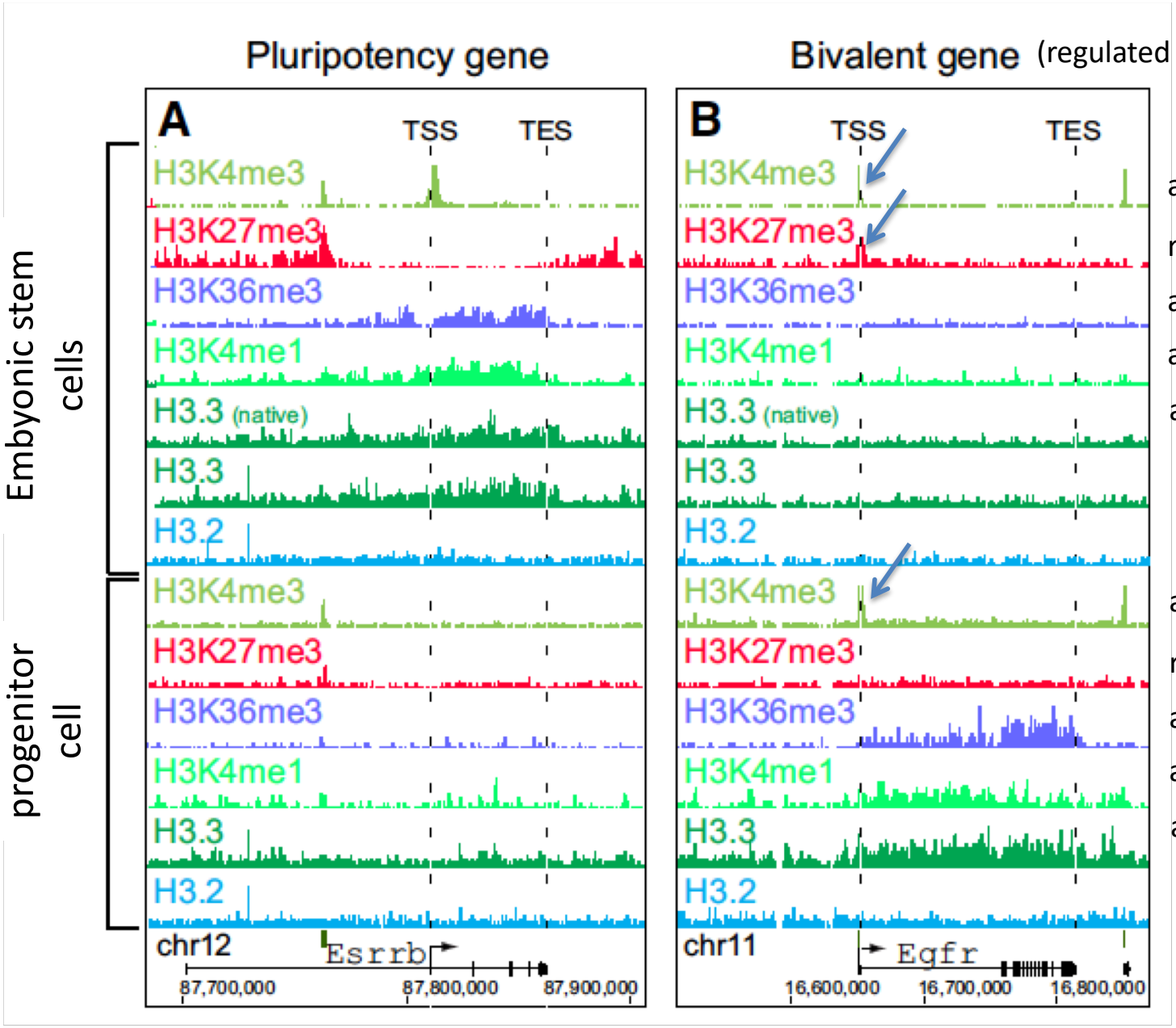


BIOINFORMATICS ANALYSIS:
Mapping ChIP seq reads agins the human genomic sequence



Goldberg et al., Cell, 140: 678-691. 2010

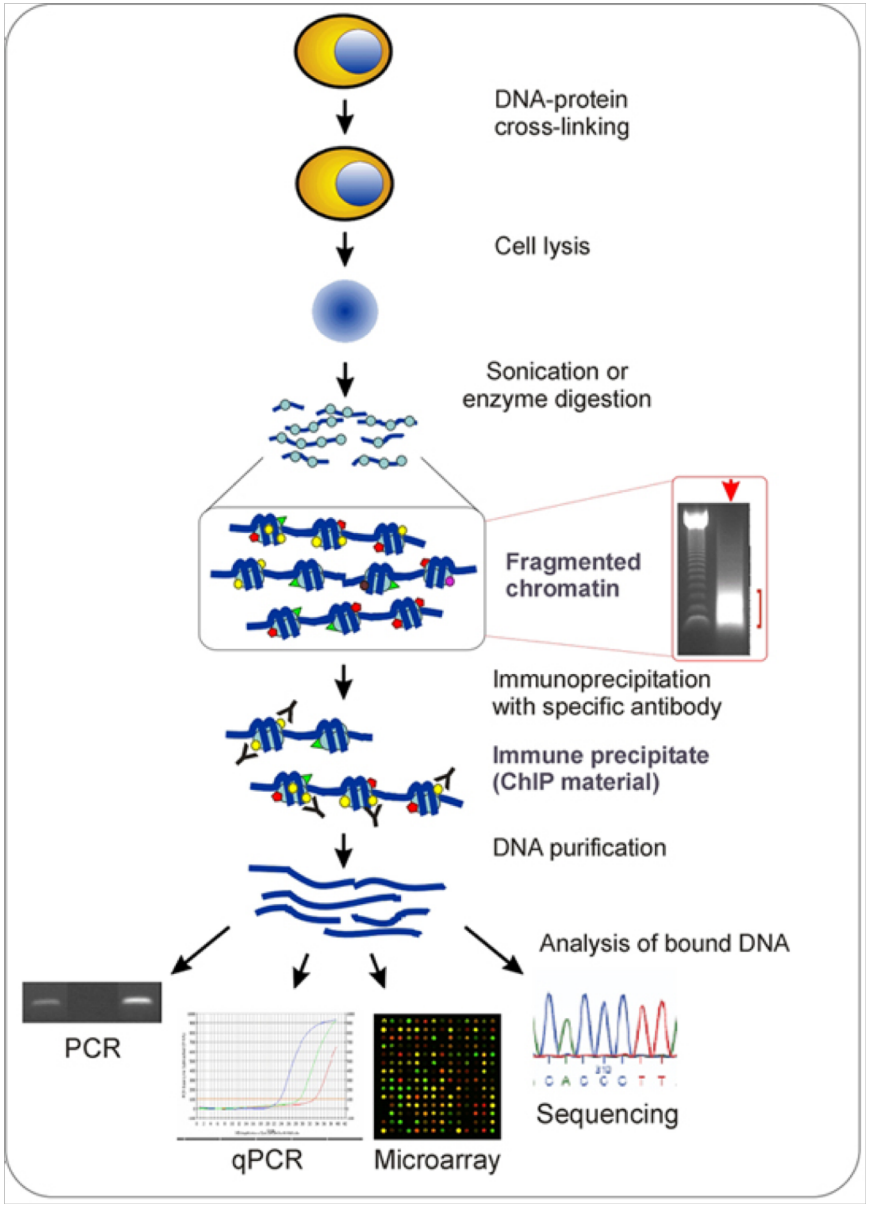
Mapping the epigenetic landscape enables to define “key rules” to define the epigenetic code of active and silent genes



Goldberg et al., Cell, 140: 678-691. 2010

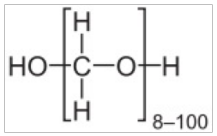
Lecture 3: Hallmark discovery and analysis of histone modifications

CHROMATIN IMMUNOPRECIPITATION → DETAILLED ANALYSIS
→ Localization of protein at a defined region - seqeunce

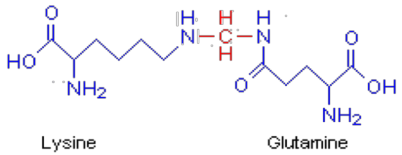


Cell model system:
i.e. Wild-type or Suv39 dn cells that grow in cell culture dish

1. Crosslink chromatin
(treatment of cells with Paraformaldehyde



Paraformaldehyde (PFA) is a polymer of formaledyde, And covalently links NH2 groups of biomolecules



Example of crosslinking reaction

2. Sonicate crosslinked cells
→DNA breaks in a random manner
until reaching a size of ca. 250 – 500nt

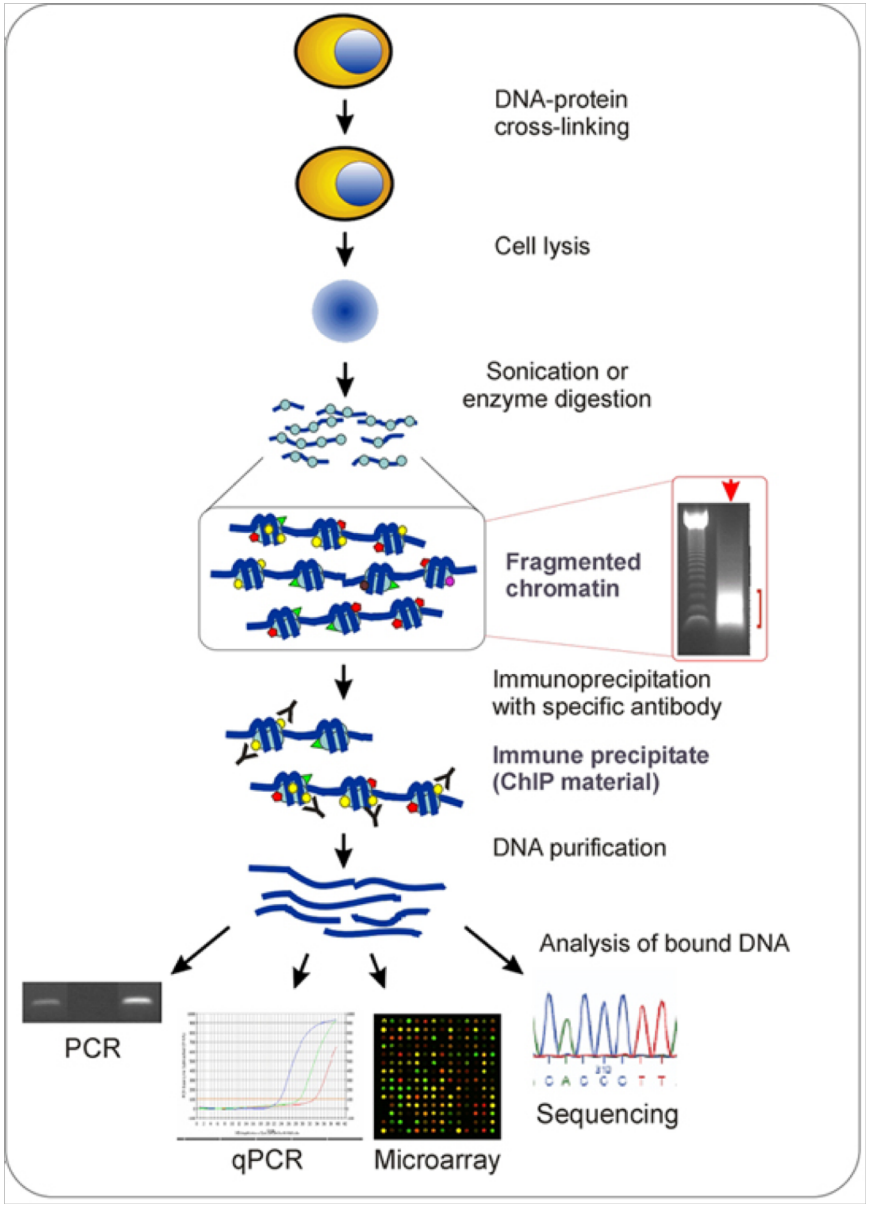
3. Incubate chromatin fragments with antibodies
that are specific for modified histones (i.e. H3K9me3) or chromatin modifying enzymes (i.e. Suv39h1); another sample with a non-specific, control antibody

4. Recover antibody-chromatin complexes using a resin that binds to the constant region of antibodies

5. Elute chromatin at high salt concentration and revert crosslinks at high temperature

6. Digest protein with protease K and RNA with RNase

CHROMATIN IMMUNOPRECIPITATION (ChIP) → DETAILLED ANALYSIS
→ Localization of protein at a defined region - seqeunce



5. Elute chromatin at high salt concentration and revert crosslinks at high temperature

6. Digest protein with protease K and RNA with RNase

7. Purify DNA and precipitate DNA

8. Measure the amount of immunoprecipitated DNA In control versus Suv39h dn cells

METHODS:

A. PCR: design PCR oligos to amplify defined sequences in immunoprecipitates. Per PCR reaction only one locus can be examined by real-time PCR

B. ChIP on ChiP:
Immunoprecipitated DNA from control and Suv39h dn cells are labeled with nucleotides that are fluorescently labeled (555nm emission fluorofoer or 488 nm emission fluorphore. Samples are mixed and hybridized to an array that contains spotted DNA probes that cover a large fraction of the genome. 488/555 nm ratio is measured → relative enrichment of histone modification at thousands of genomic sites in control/Suv39h dn cells

B. ChIP Seq:
Immunoprecipitated DNA is sequenced by massive parallel Sequencing. Control vs. Suv39h dn cells reveals enrichment of H3K9me3 at the ENTIRE genome level.
single nucleotide levels