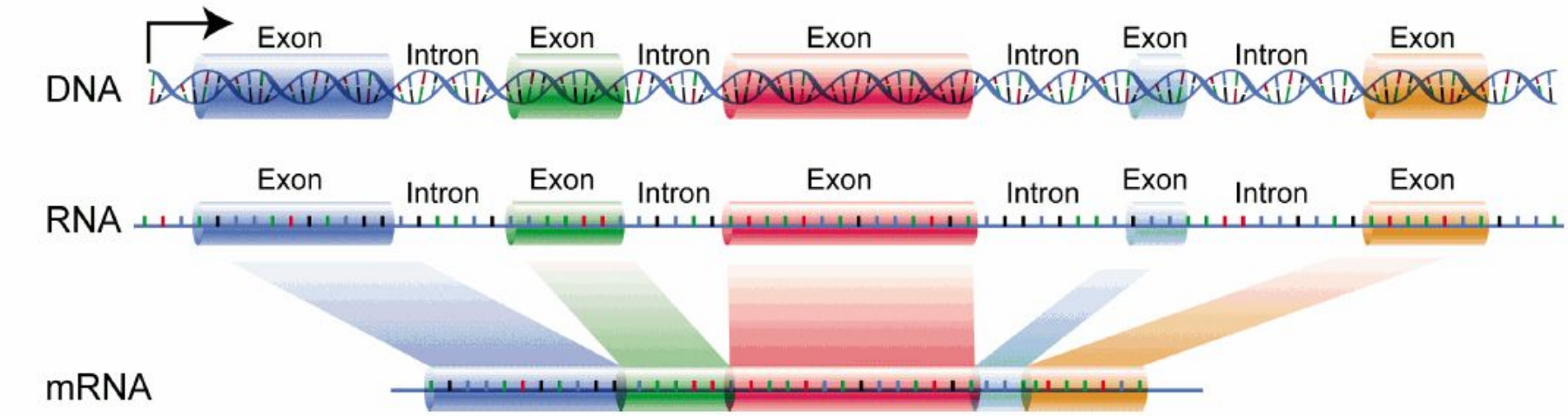
Variant Calling and Clustering on RNA-Seq Data

by Paul Arndt and Karsten Tausche
03.02.2017

Agenda

- The basics
- History of DNA Sequencing Technologies
- RNA-Seq and Variant Calling Pipelines
- Clustering on mixed data
- Hands-on: Variants in practice
- Ethics discussion

The basics


DNA
Exon
Intron
Exon
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Exon
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RNA
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Exon
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Exon
mRNA

The basics

- RNA sequencing expression
  - differences in mapped reads between different samples → compare the amount of specific genes
  - quantitative data

- Variant Calling: DNA vs. RNA
  - DNA sequencing: analytically complex and not very efficient
  - RNA sequencing: cheaper, and, because of the traditionally used RNA sequencing expression analysis, the data is already there
    - → Variant Calling on RNA Data
    - but: beware that RNA only contains genes expressed in the analyzed cells, not the whole genome

The basics

Variants

→ differences in genes, according to a reference genome

- SNPs (Single Nucleotide Polymorphisms) vs CNVs (Copy Number Variant)
- Risk Variants vs Protective Variants

History of Genetics

Relatively short history is basis for our current understanding

- 1869: Nucleic acid
- 1919: Polynucleotide model: four bases, sugar, phosphate
- 1944: Genes
- 1954: Structure of the Deoxyribonucleic acid (DNA)
- 1984: Initial Idea of the “Human Genome Project”
- 2000: First Draft of HG
- 2003: HG completely sequenced

DNA Sequencing Technologies

Human Genome: 3.2 Gbp (Million basepairs)

- First Generation Sequencing (ABI 2002): *Human Genome Project*
  - Very high accuracy (> 99.99%)
  - Slow processing (1 run = 100kbp, 3h)
- **Next Generation Sequencing: Illumina (2006): *Today's Standard***
  - Acceptably high accuracy (> 99.9%)
  - 2006: 1Gbp / run, 2016: 1 Tbp / run (6 days)
  - **Short read length**: **200-400bp, later up to 700bp → fragmented output!**
- Pacific Biosciences: Third Generation Sequencing (2013)
  - Long read sequencing: 60kbp ("DeNovo Alignment")
  - Accuracy > 99% (!)

Illumina Sequencing Process (simplified)

1) Preparation
   - Fragmentation of DNA into chunks (“reads”)
   - Required to be able to read sequence
   - 200-800 bp (3.2 Gbp in Human Genome!)
2) Amplification
   - Generate readable DNA regions (clusters)
3) Sequencing
   - Light reflected differently by each nucleotide
   - Record laser light reflection image
   - Generate textual output from recorded image

[https://www.illumina.com/systems/array-scanners/nextseq-550.html, illumina, 2017]

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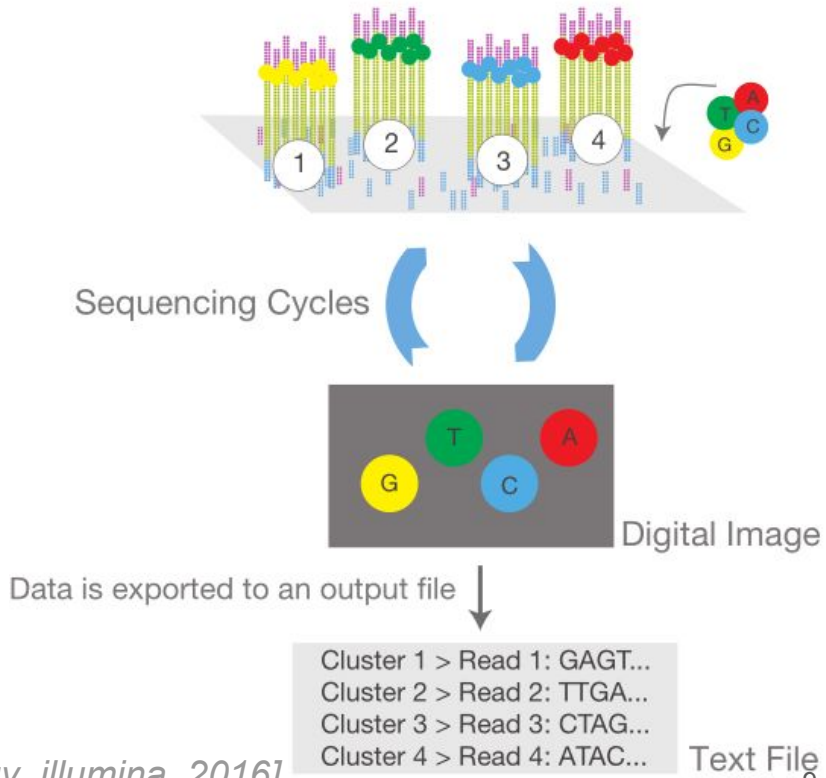
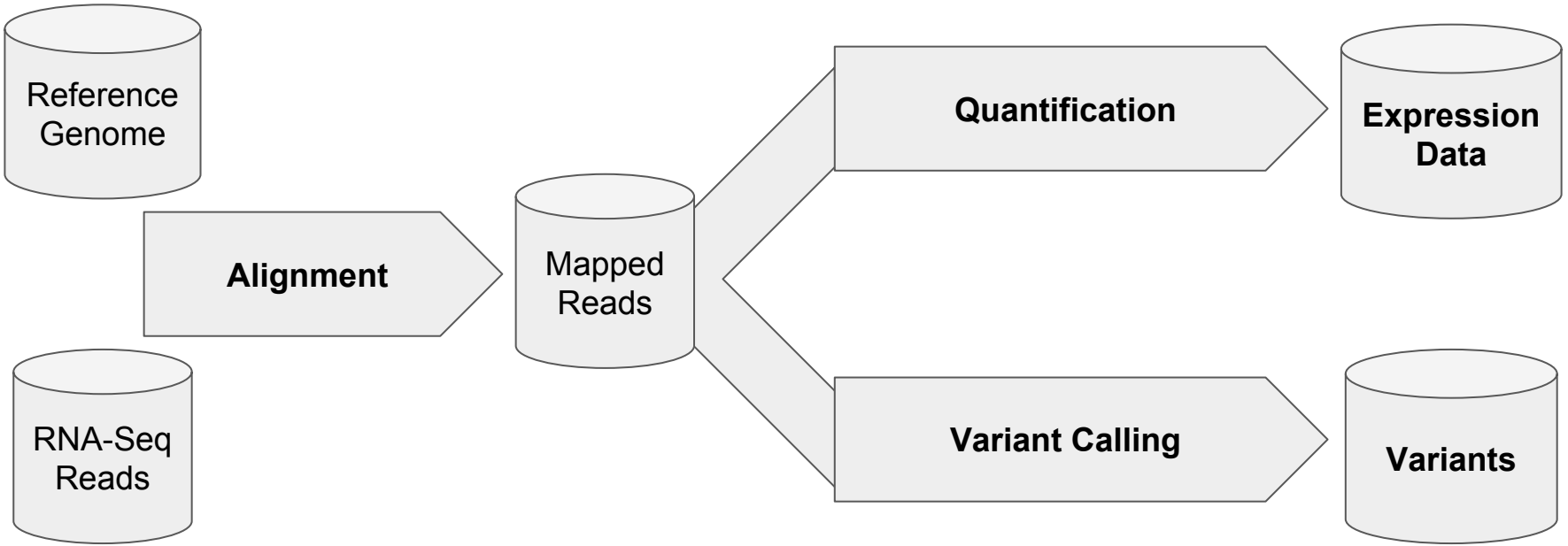


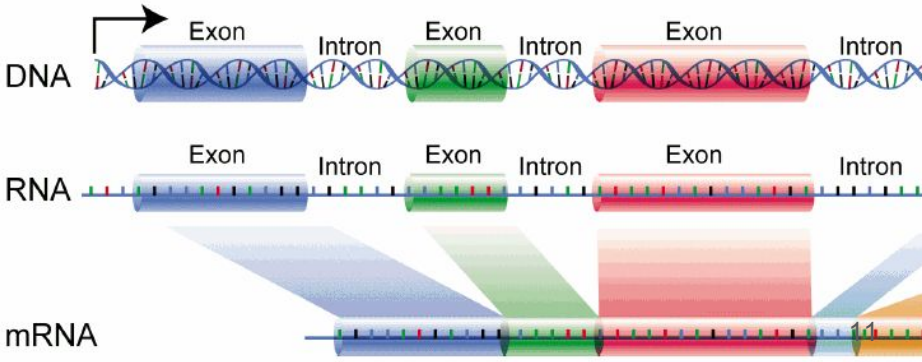
[An Introduction to Next-Generation Sequencing Technology, illumina, 2016]

RNA-Seq based Pipelines

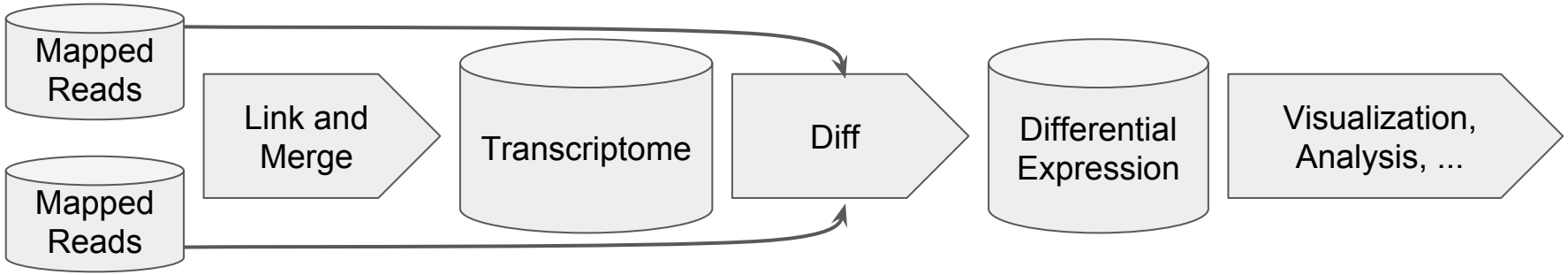


Short Read Alignment

- Sequenced RNA: Many small RNA chunks (reads)
- Locate related position in the reference genome
  - Could be anywhere in the coding regions
  - Many highly similar regions within the DNA
  - Related coding DNA part may contain non-coding (irrelevant) parts
  - Editing events occur at specific regions/genes
- Process aligned reads
  - Probably many reads for same locations
  - Partly overlapping reads
  - Contradictory information
  - Apply statistical methods

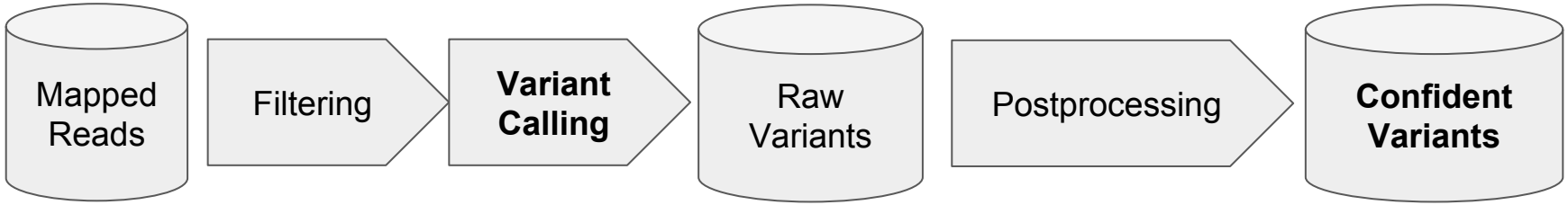


Proteine Quantification Pipeline



- Multiple input samples (e.g., two conditions, healthy, ill)
- **Transcriptome**: set of all mRNA in a cell
  (≈ genes expressed in that cell)
- **Differential Expression**
  Differences of mRNA quantities between the samples

RNA-Seq based Variant Calling



- Filtering
  - Deduplication
  - Remove low-quality reads (defined by sequencing device)
  - Filter unmapped reads
  - Filter low quality reads/mappings
- Variant Calling
  - Find deviation from reference genome
- Postprocessing
  - Separate Variants from Indels
  - Filter low-quality variants
  - Filter false-positive variants

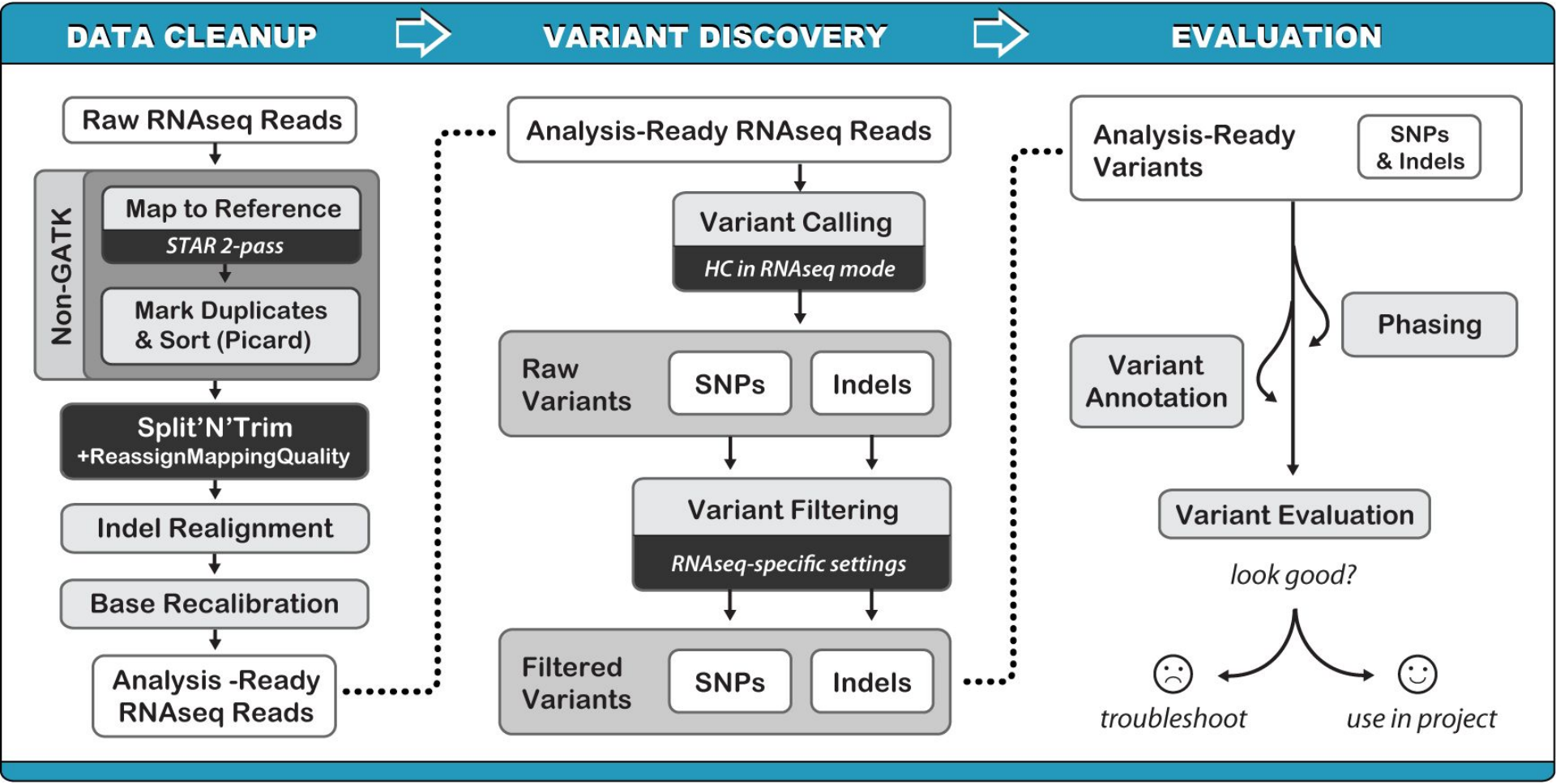
RNA-Seq based Variant Calling Pipelines

SNPiR: “Reliable Identification of Genomic Variants from RNA-Seq Data” [Piskol 2013]

- High sensitivity
  - Loose criteria in variant calling step
- High specificity
  - Extensive filtering to omit false-positives
- Based on tools optimized for DNA-Seq Data

GATK Best-Practices: “Calling variants in RNAseq” [2014-2017]

- Built on newer tools, specialized for RNA-Seq Data
- Including some concepts of SNPiR



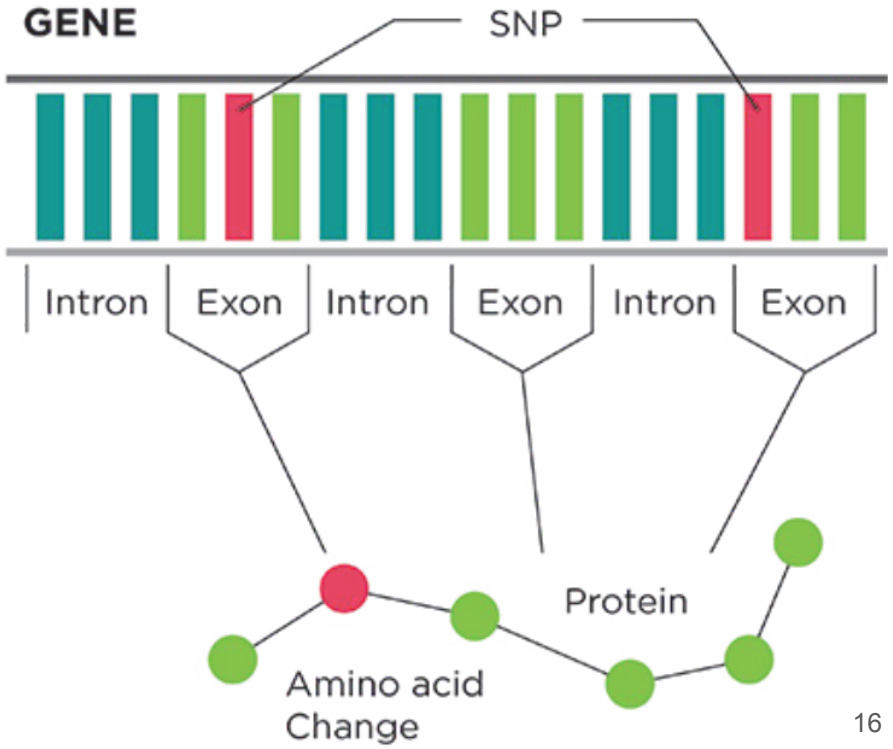
[https://software.broadinstitute.org/gatk/guide/article?id=3891 2017]

Alignment Across Splice Junctions

- Genome consists of **exons** (coding) and **introns** (non-coding)
- **Splicing**: removal of introns, joining of adjacent exons

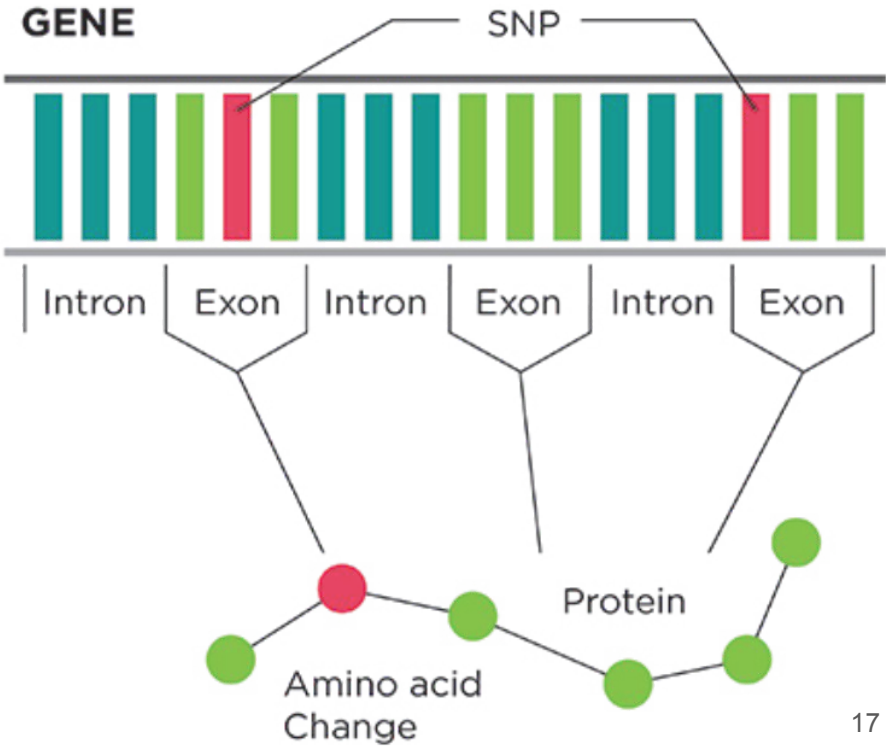
- Not all **splice junctions** are known

- How to align reads across splice junctions?



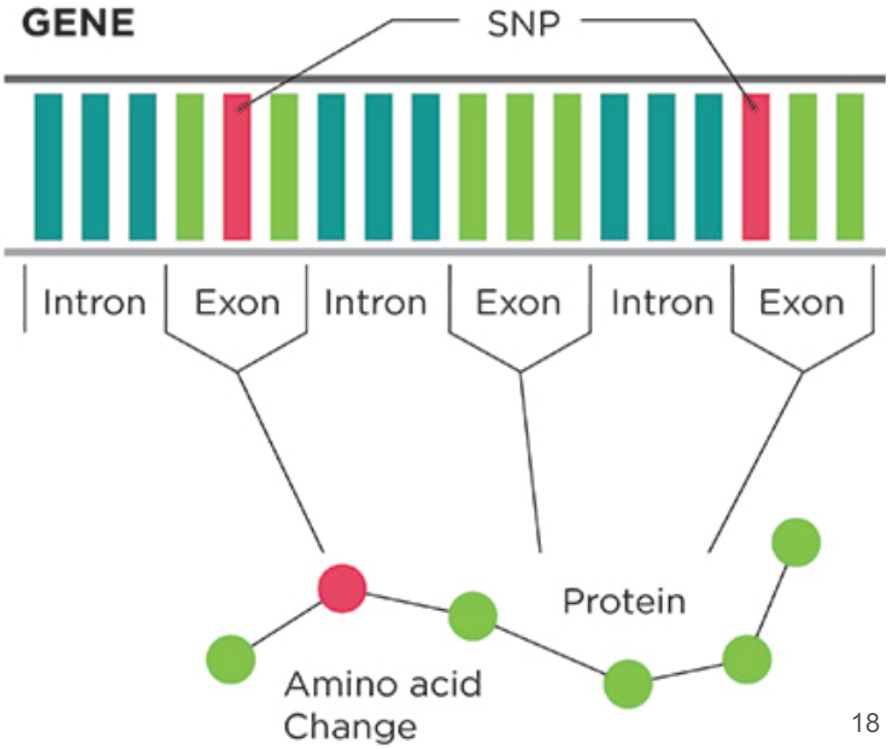
Alignment Across Splice Junctions

- Alignment to genome only?
  - Algorithm would probably find a similar (wrong) location
- Alignment to transcriptome only?
  - Transcriptome may not be complete
- Combined approach!
  - Align to Genome
  - + known parts of the transcriptome



GATK: Two-Pass Alignment

- Using **STAR** aligner
  - State-of-the-art for RNA-Seq data
- Option: “2-pass STAR”
  - Detect splice junctions in first run
  - Use generated information in second run → final alignment
- Not using previously known splice junctions
  - No additional data dependencies
  - Missing information?



Filtering based on Genome Annotation

[USCS Genome Browser: Genomes + Annotations]

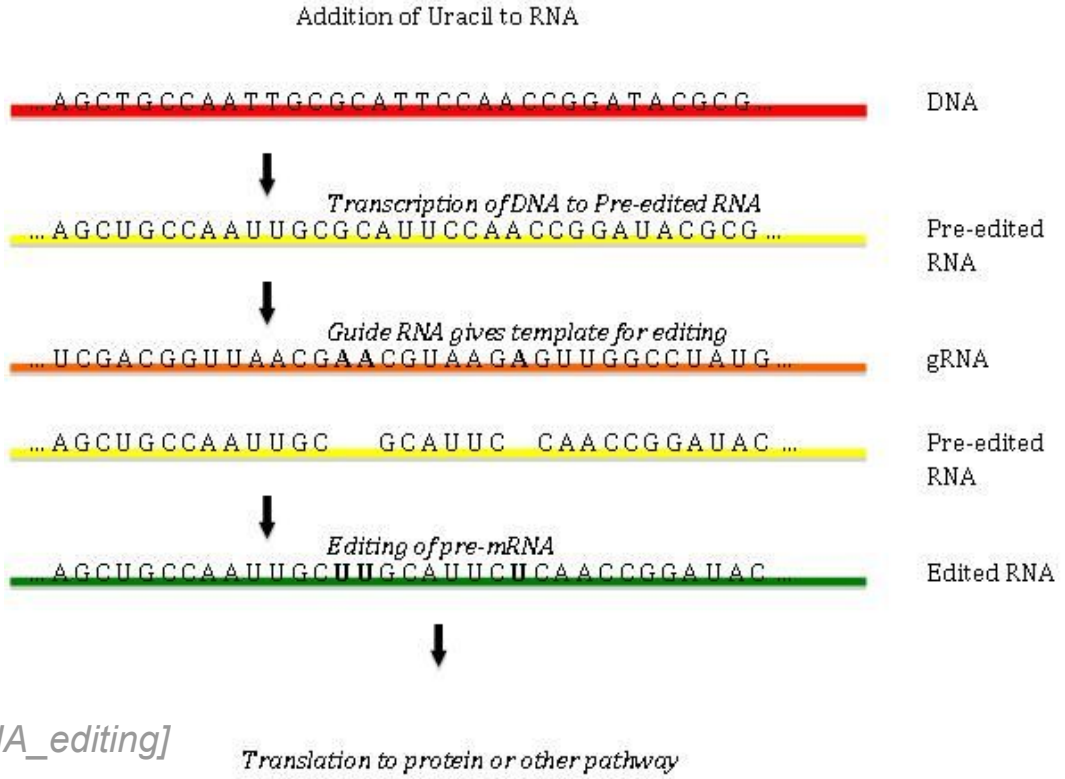
RepeatMasker Annotation

- Genome contains highly repetitive regions
- Controlling transcriptions, immunity against foreign DNA, …
- Generally non-coding
- Difficult/impossible to correctly align reads to

Filtering based on Genome Annotation

RNA Editing Sites

- Nucleotide sequence differs from original sequence in DNA
- Complicates read alignment
- Differences must not be interpreted as variants



[https://en.wikipedia.org/wiki/RNA_editing]

Filtering based on Genome Annotation

- Heavily used by SNPiR
  - Pseudo-Chromosomes
  - Post-processing after variant calling
- Not part of the GATK-Pipeline
  - Relying on advanced, specialized tools
  - Not relying on previously known data
- Apply SNPiR filtering to GATK-Pipeline?
  - Focus on human genome: rich information available
  - Filtering most reliable variants based on all known data

Statistical Filtering Strategies

- Statistical decisions in whole pipeline
  - Quality scores for alignment (depth, certainty)
  - Quality scores for called variant
  - Uncertainties in reference genome, two DNA strands, …
- Quality score evaluation requires reference scores
  - “Base quality score recalibration”
  - Data available for DNA-Seq
  - Not yet available for RNA-Seq
- Evaluation using known DNA-Seq variants
  - Currently most reliable way to verify tools and pipelines

Raw Sequencing Data: FASTQ Files

@SRR831012.1 HWI-ST155_0742:7:1101:1284:1981/1
NGAGATGAAGCACTGTAGCTTGGAATTCTCGGGTGCCAAGGAACTCCAGT
+
%1=DDDFFHHHGFIHHIIIIIIIIIIIIIIIIIEHIIIIIIIFIIIIIII

@SRR831012.2 HWI-ST155_0742:7:1101:2777:1998/1
NGAGATGAAGCACTGTAGCTCTTTGGAATTCTCGGGTGCCAAGGAACTCC
+
%1=DFFFFHHHHHIIIIIIIIIIIIIIIIIIGIIIIIIIIIIIIIIIIG

Quality score (increasing from worst to best):
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNOPQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~

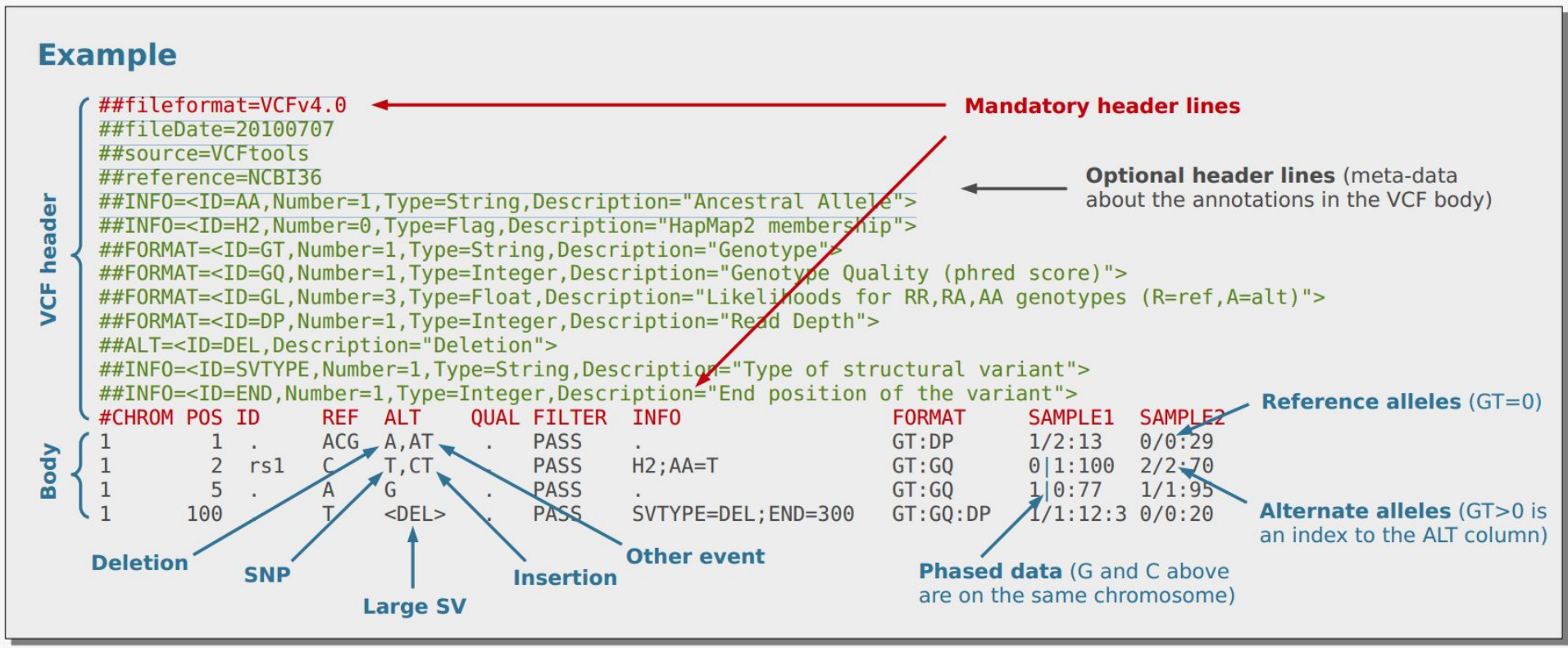
@SampleID.ReadNr

Experimental Setup

In our setting:

- ~1.4 GB per file
- ~8 Mio reads per file
- 80 files

VCF: Variant Call Format



[Vishuo Pte Ltd, 2014, http://vishuo.com/new/the-variant-call-format-and-vcftools]

How to make sense of the data

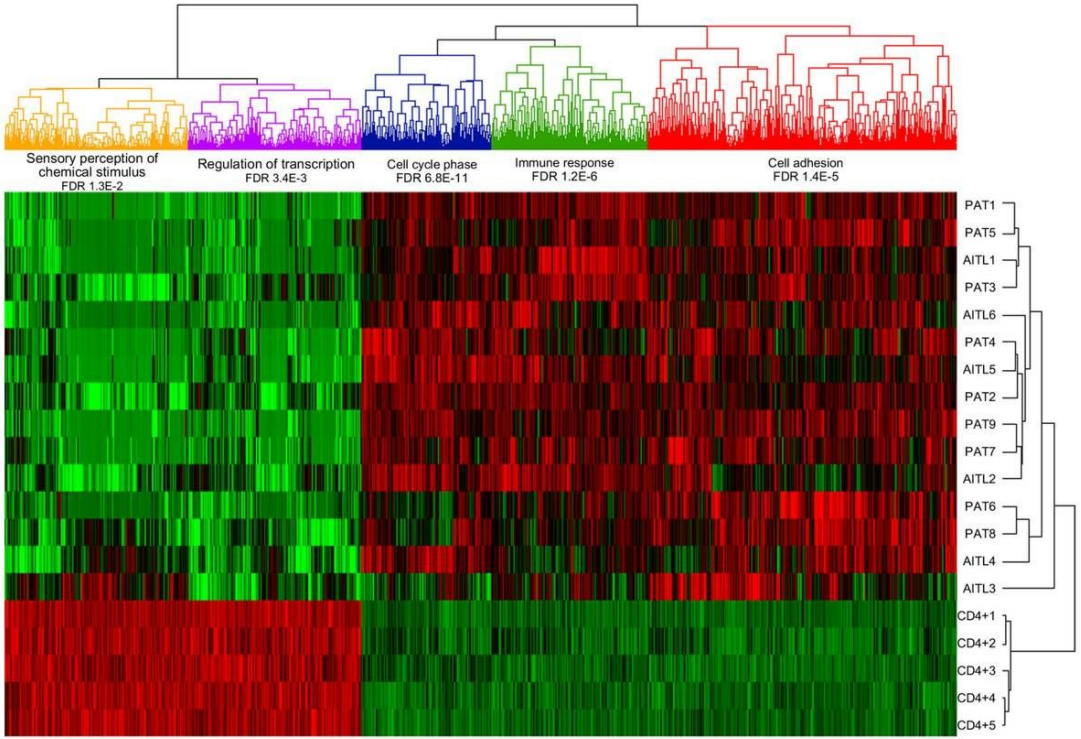
open question: What do newly sequenced genes do?

- infer correlations between different genes - allowing for example the building of classifiers to improve diagnosis, ...

other general use cases for clustering in bioinformatics:

- complexity reduction

How to make sense of the data



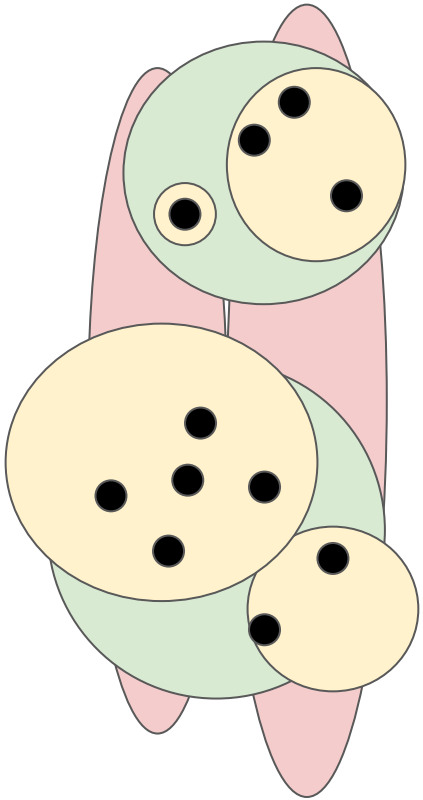
Clustering

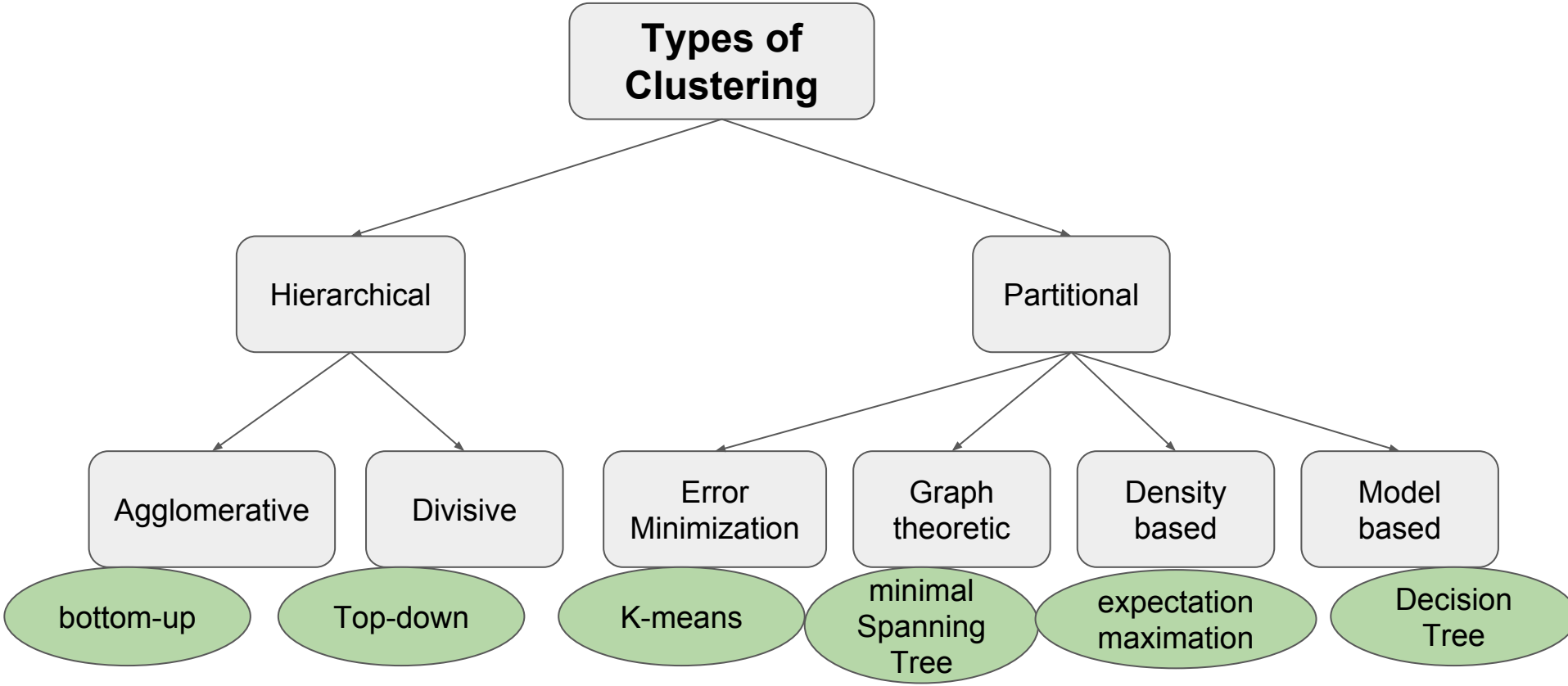
Main Principles: Homogeneity, Separation

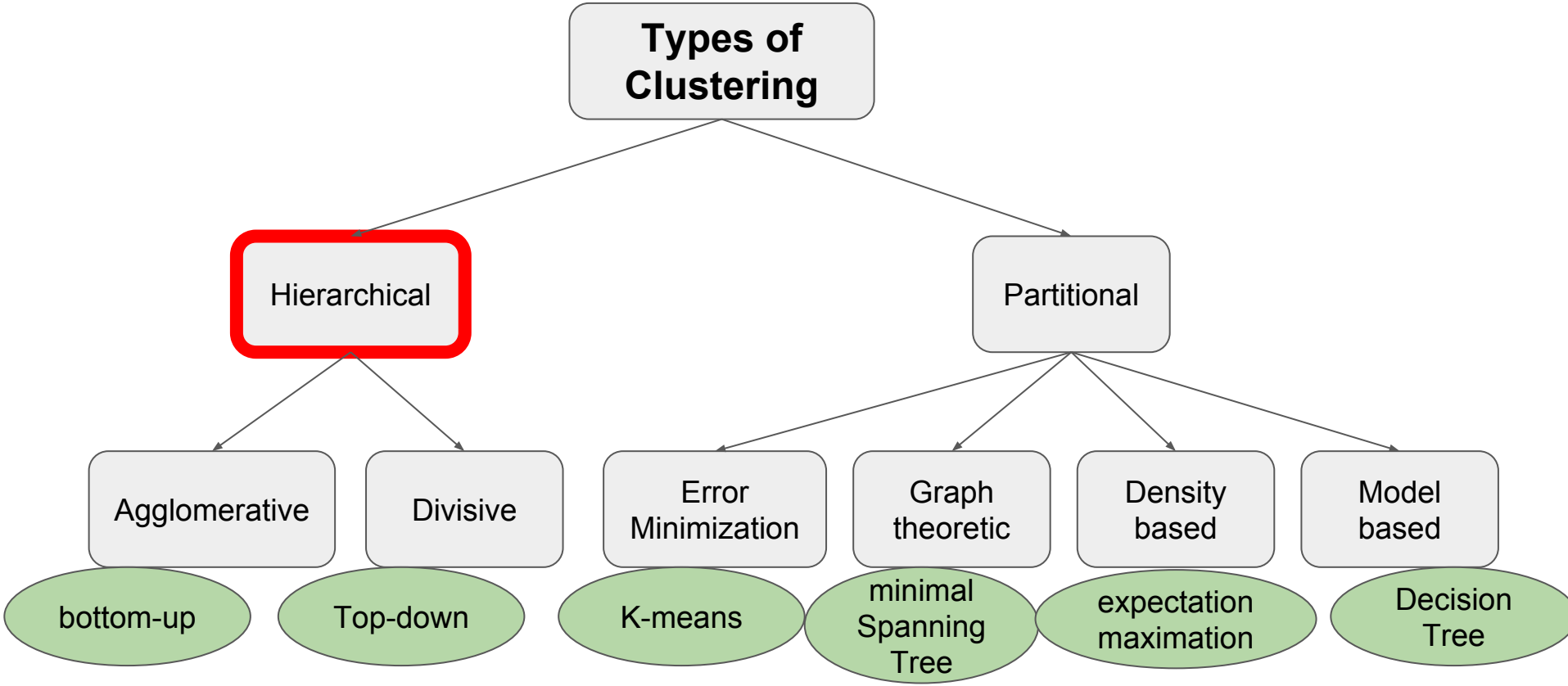
very intuitive for us in 2-D

Problem: n-dimensional data

- curse of dimensionality




Types of Clustering
Hierarchical
Partitional
Agglomerative
Divisive
Error Minimization
Graph theoretic
Density based
Model based
bottom-up
Top-down
K-means
minimal Spanning Tree
expectation maximation
Decision Tree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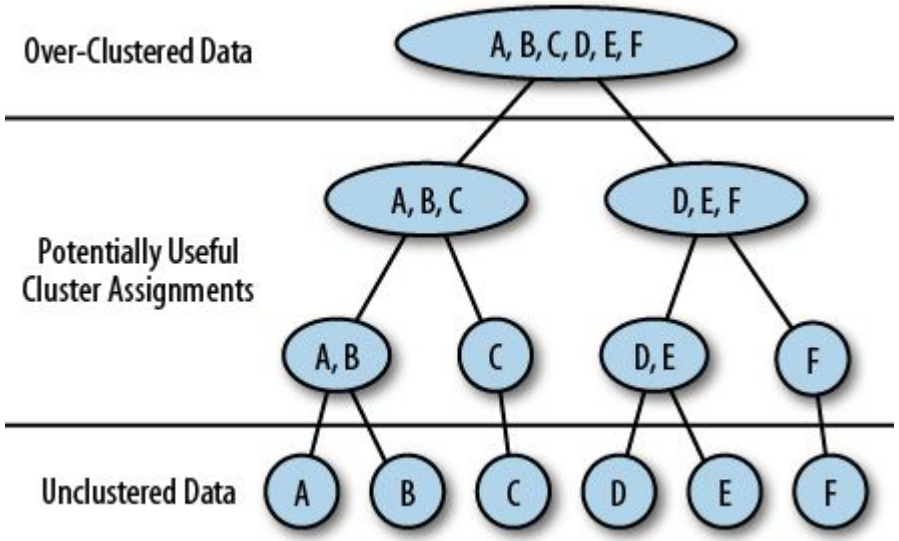
Example Hierarchical Clustering

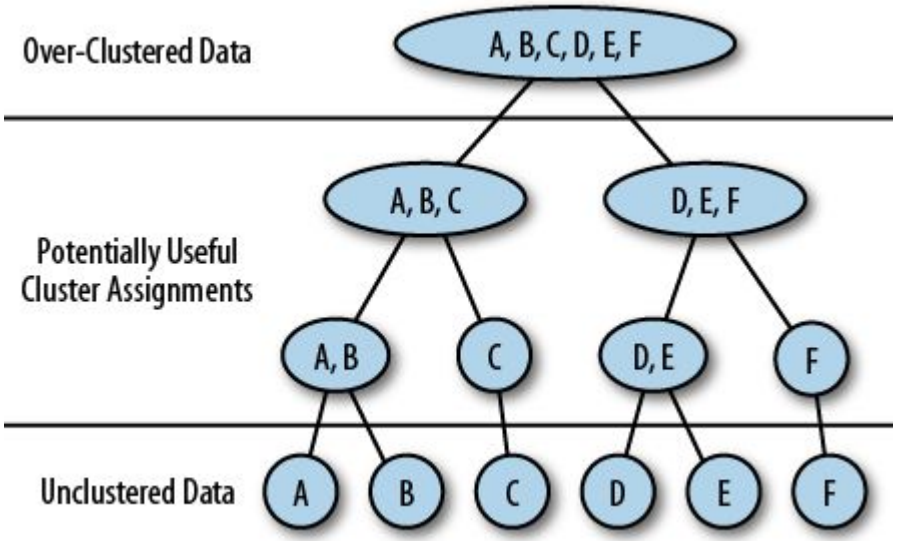
1. Every node is assigned its own cluster
2. Find the closest pair of nodes and merge them into a cluster
3. Repeat step 2, until all nodes in the network have been merged into a single large cluster
4. Choose a useful clustering threshold between the bottom and top levels

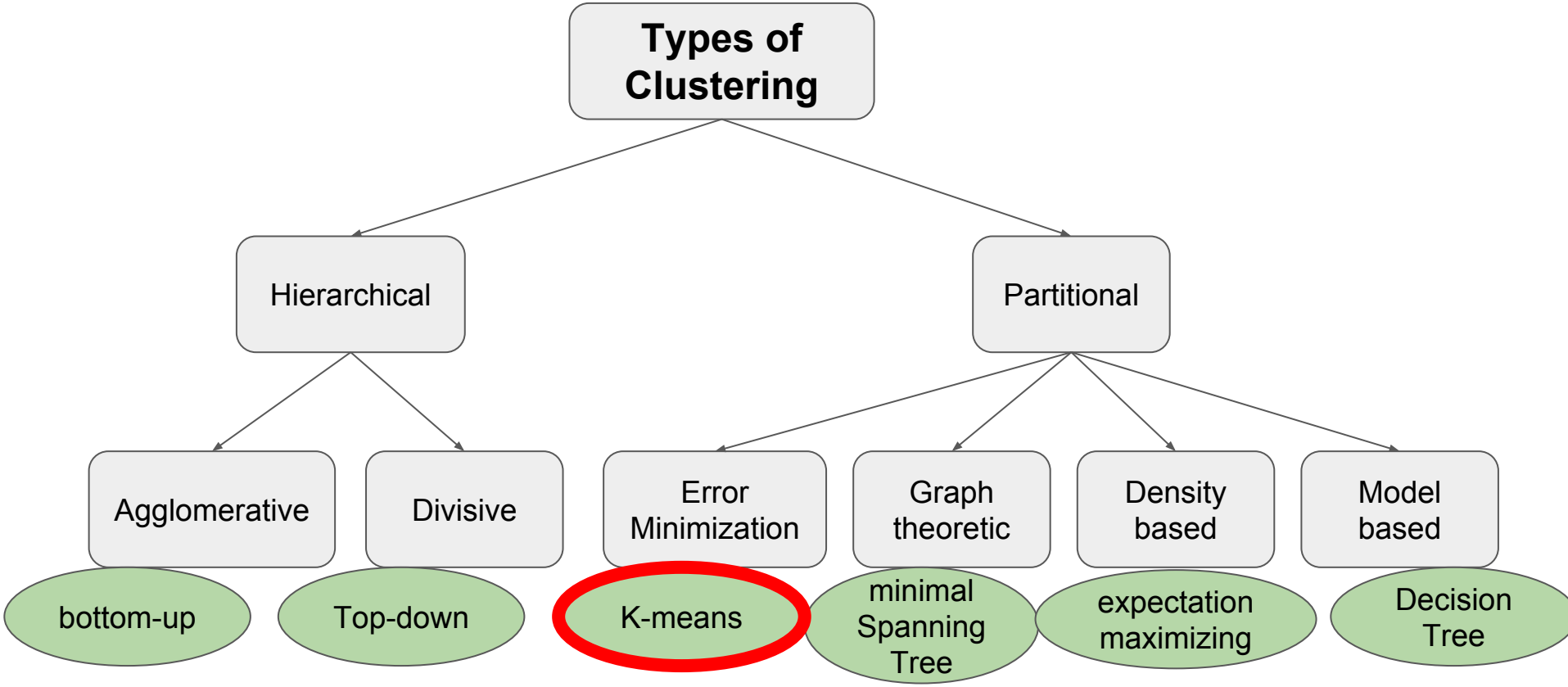


Example Hierarchical Clustering

How do you compute the distance between clusters?

- Single-link: merge two clusters with the smallest **minimum** pairwise distance
- Average-link: merge two clusters with the smallest **average** pairwise distance
- Maximum-link or Complete-link: merge the two clusters with the smallest **maximum** pairwise distance

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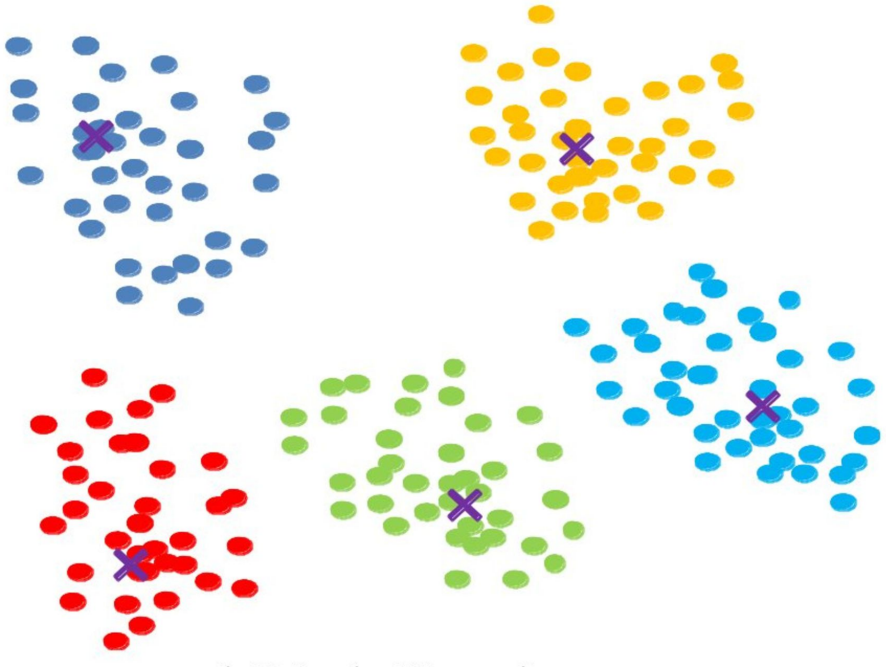
Example K-means

The main idea is to define k centroids, one for each cluster.

1. Select k entities as the initial centroids
2. (Re)Assign all entities to their closest centroids
3. Recompute the centroid of each newly assembled cluster
4. Repeat step 2 and 3 until the centroids do not change or until the maximum value for the iterations is reached



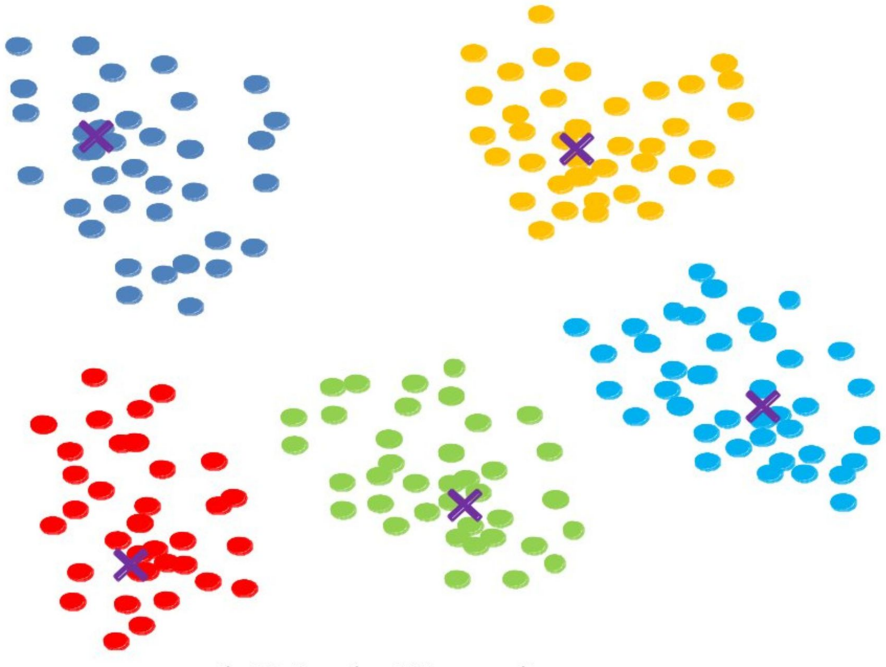
Example K-means

advantages:

- simple, fast, efficient (O(n))

disadvantages:

- difficult to predict K, often produces clusters of uniform size, spherical assumption



Handling Mixed Data

Clustering so far is almost exclusively done on quantitative data

Now: adding Variants (qualitative data) → mixed Data

Main Problem: How to compute distances?

Clustering - Distance measures

COR Pearson sample metric

EISEN Cosine correlati

SPEAR Spearman sample correlation distance

$$d_{spear}(\mathbf{x},\mathbf{y}) = 1 - \frac{\sum_{i=1}^{m}(x'_i - \overline{x'})(y'_i - \overline{y'})}{\sqrt{\sum_{i=1}^{m}(x'_i - \overline{x'})^2 \sum_{i=1}^{m}(y'_i - \overline{y'})^2}}$$

$$\frac{\sum_{i=1}^{m}(x_i - \overline{x})(y_i - \overline{y})}{\sqrt{\sum_{i=1}^{m}(x_i - \overline{x})^2 \sum_{i=1}^{m}(y_i - \overline{y})^2}}$$

Gower Similarity

compares two cases i and j

- Sijk: contribution provided by the k-th variable
- wijk: 1 or 0 depending on the comparison

basically case distinction depending on variable type

$$S_{ij} = \frac{\sum_{k}^{n} w_{ijk} S_{ijk}}{\sum_{k}^{n} w_{ijk}}$$

Gower Similarity

$$S_{ij} = \frac{\sum_{k}^{n} w_{ijk} S_{ijk}}{\sum_{k}^{n} w_{ijk}}$$

ordinal/continuous variables:

$$S_{ijk} = 1 - \frac{|x_{ik} - x_{jk}|}{r_k}$$

rk is range of values for the k-th variable

Gower Similarity

$$S_{ij} = \frac{\sum_{k}^{n} w_{ijk} S_{ijk}}{\sum_{k}^{n} w_{ijk}}$$

nominal variables: Sijk = 1 if Xik = Xjk or 0 if Xik != Xjk

wjk = 1 if both cases have observed states for k

Gower Similarity

binary values

	Value of attribute k			
Case *i*	+	+	−	−
Case *j*	+	−	+	−
S_{ijk}	1	0	0	0
w_{ijk}	1	1	1	0

$$S_{ij} = \frac{\sum_{k}^{n} w_{ijk} S_{ijk}}{\sum_{k}^{n} w_{ijk}}$$

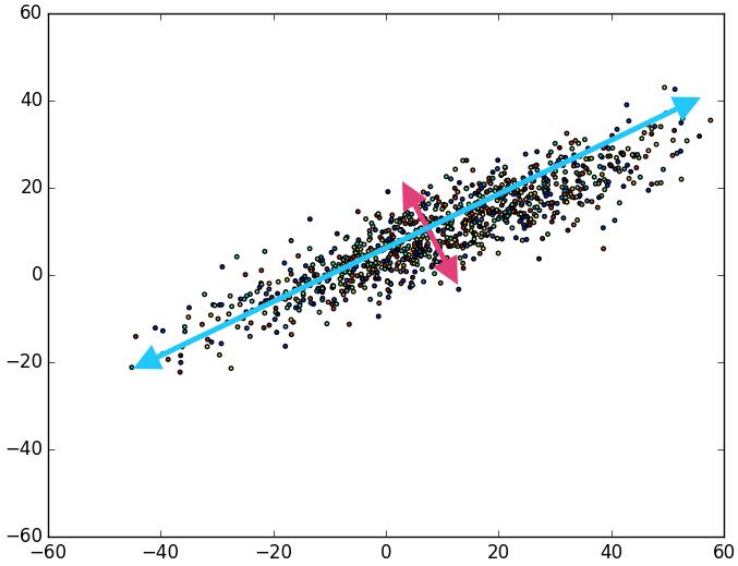
Multiple Factor Analysis

It may be seen as an extension of:

- Principal component analysis (PCA) when variables are quantitative,
- Multiple correspondence analysis (MCA) when variables are qualitative,
- Factor analysis of mixed data (FAMD) when the active variables belong to the two types.

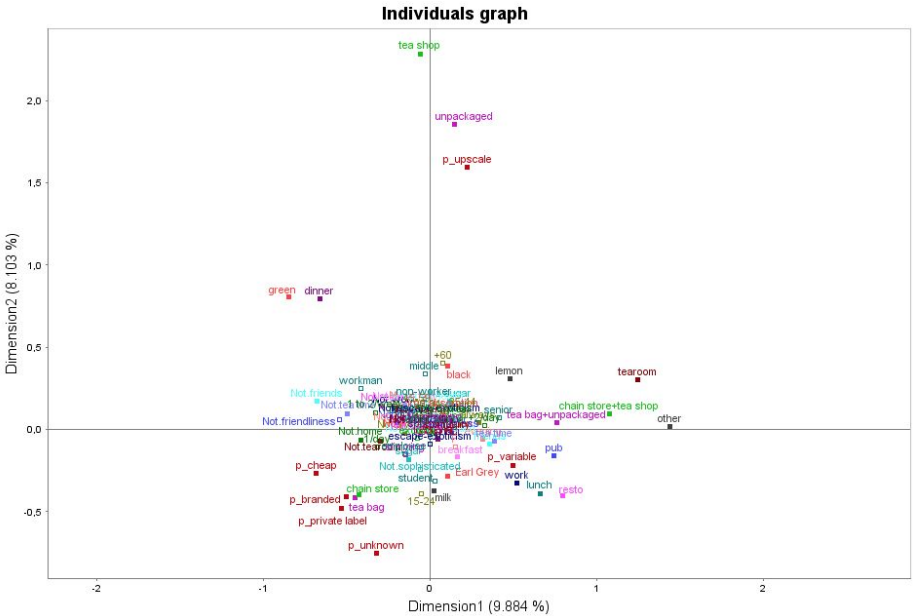
Multiple Factor Analysis

PCA

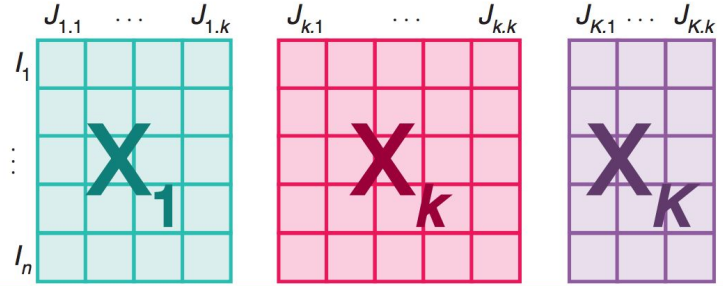


MCA: also a dimension reducing method; it represents the data as points in 2- or 3-dimensional space.

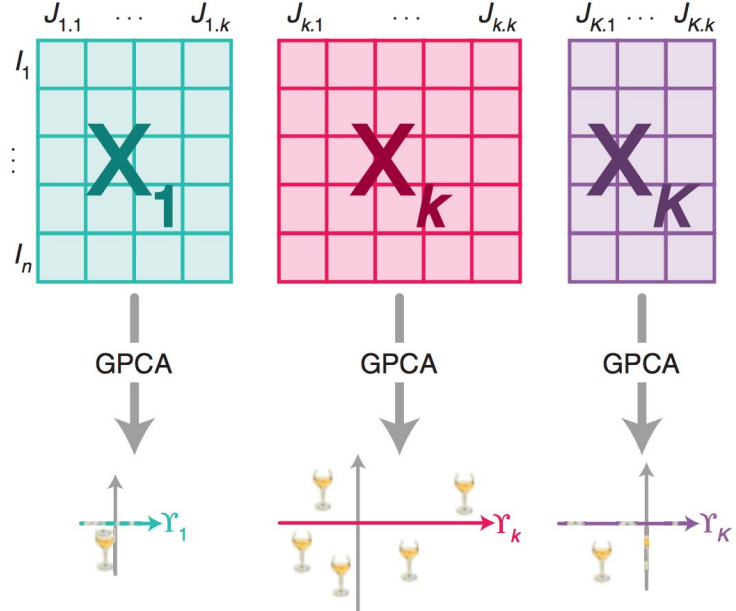
indicator matrix or burt table



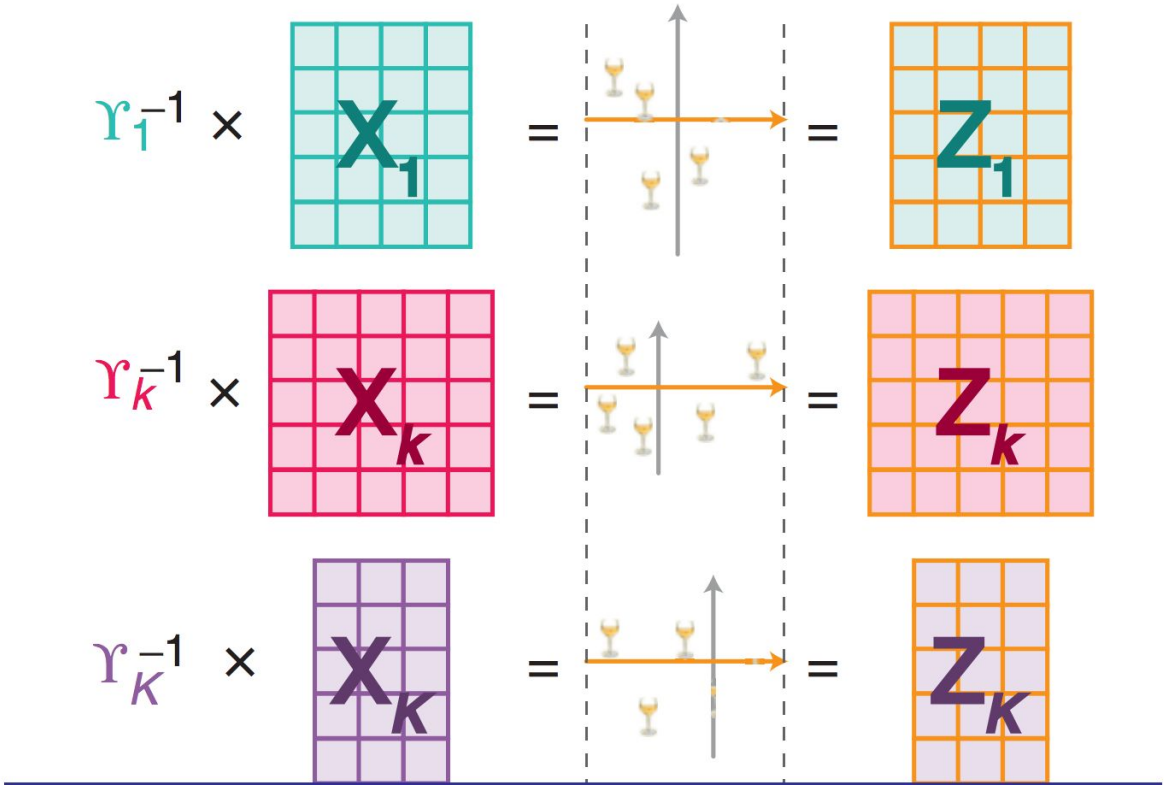
Step 1: K tables of J_k variables collected on the same observations



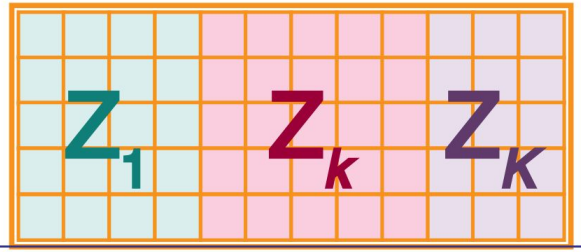
Step 2: Compute generalized PCA on each of the K tables (where Υ is the first singular value of each table)



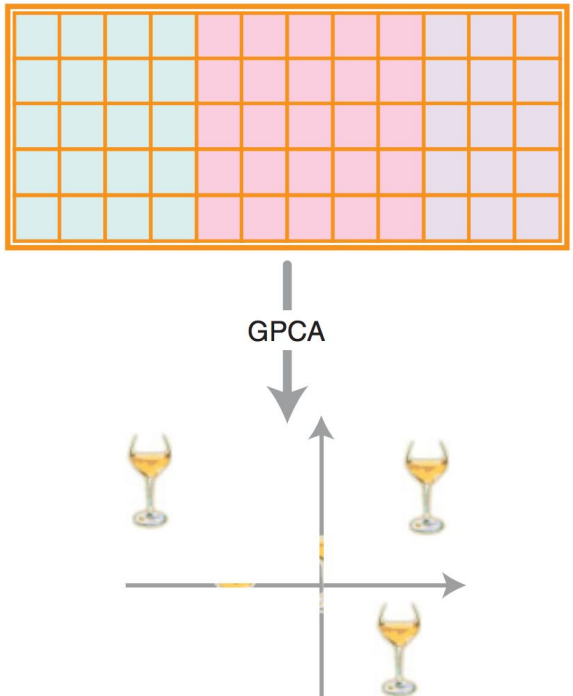
Step 3: Normalize each table by dividing by its first singular value (Υ)



Step 4: Concatenate the *K* normalized tables



Step 5: Compute a generalized PCA on the concatenated table



Clustering results - now what?

We will hopefully see some patterns that we can associate with diseases / known issues

To prove this, we can, for example, look at the Variants that got clustered together and check whether they are associated with similar problems

Hands-On: Genome Browser