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Image analysis

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To define particular question - goal

- Hypothesis confirmation
- Investigation of a particular problem

To asses the number of replicates

- to enable statistical data evaluation

Workflow of sample preparation, kind of control samples, standards

kind of detection

Biological

- Involve biological diversity
  - replicates represented by different samples of the same nature

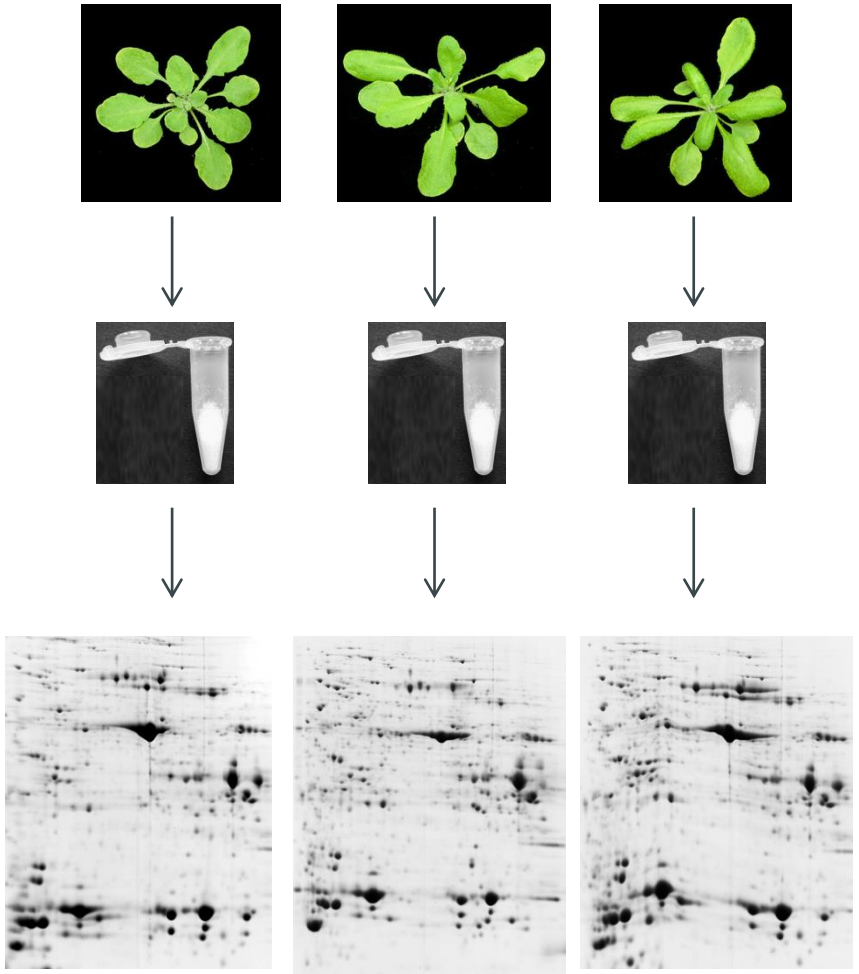
  X Arabidopsis plantlets,

  X the same type of tumour from different patients

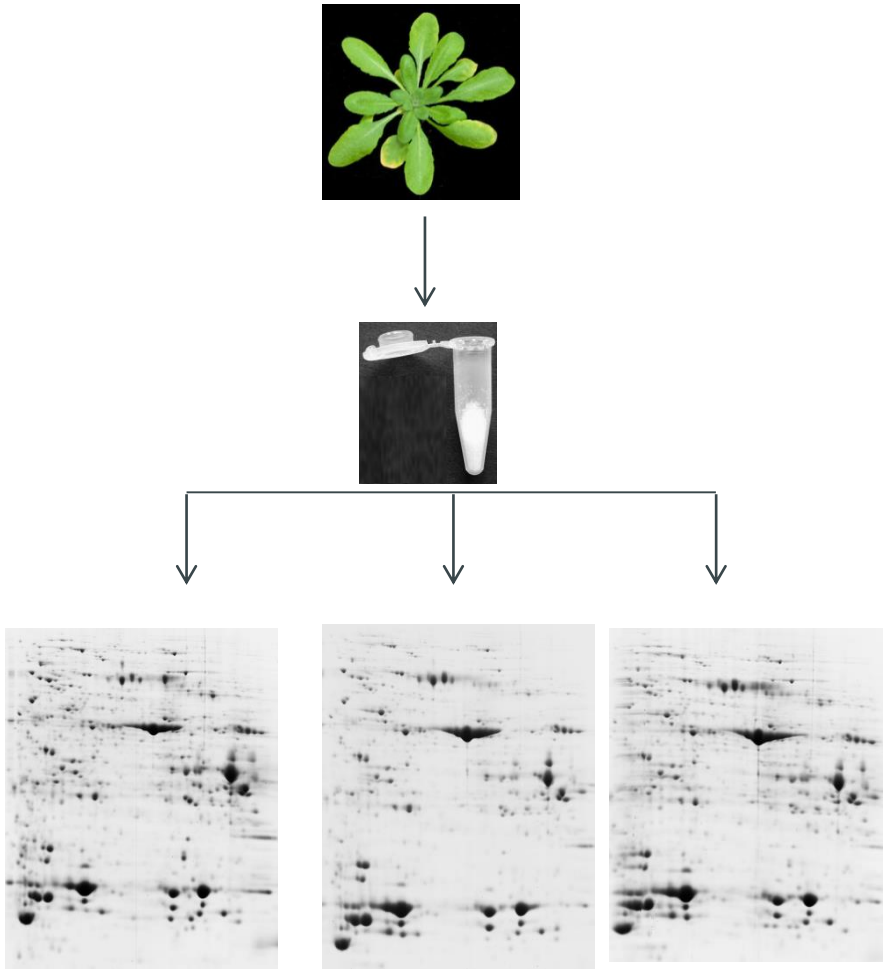
Technical

- Involve an error of the method (reproducibility)
  - identical sample in several replicates

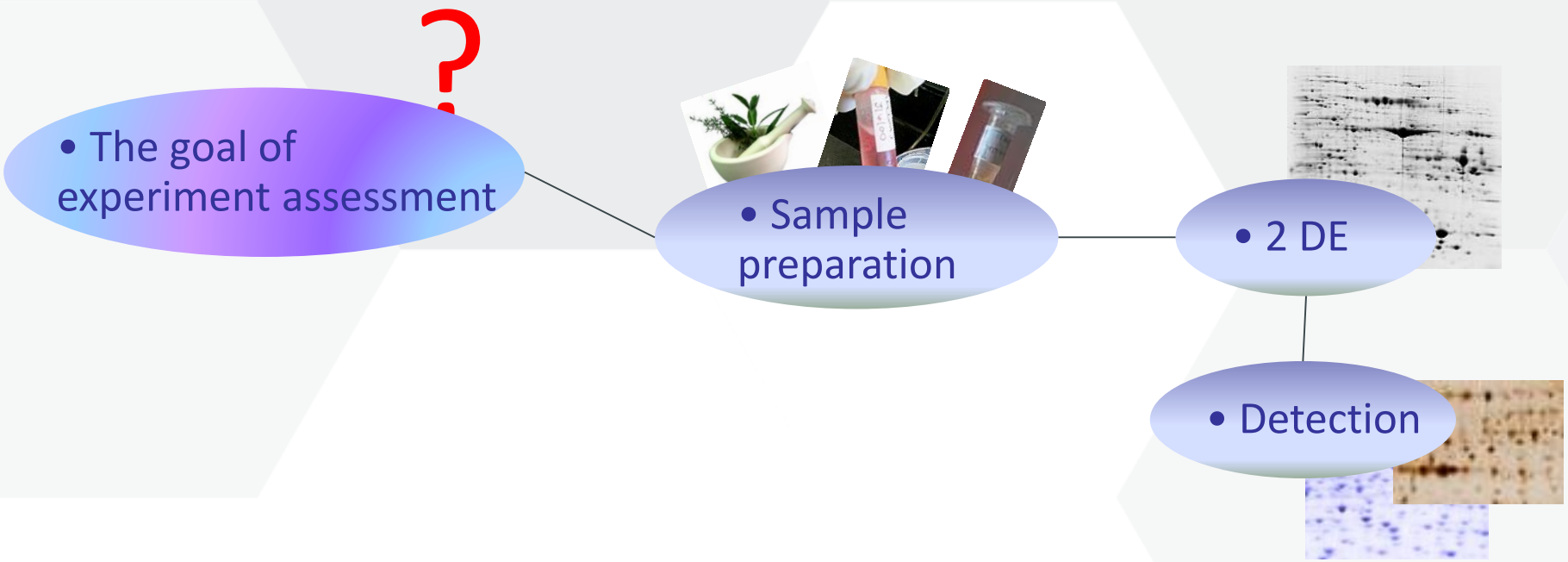
Biological replicates



Technical replicates



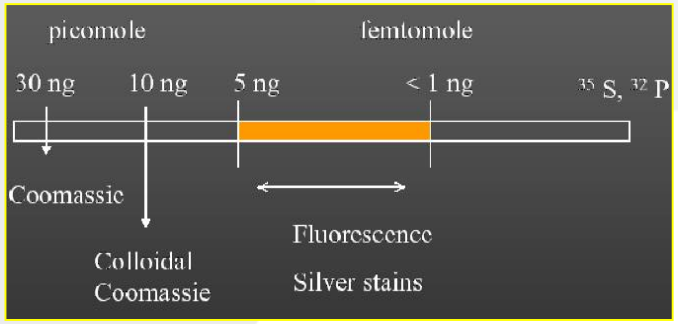
Experiment


?
• The goal of experiment assessment
• Sample preparation
• 2 DE
• Detection

General requirements on protein visualization

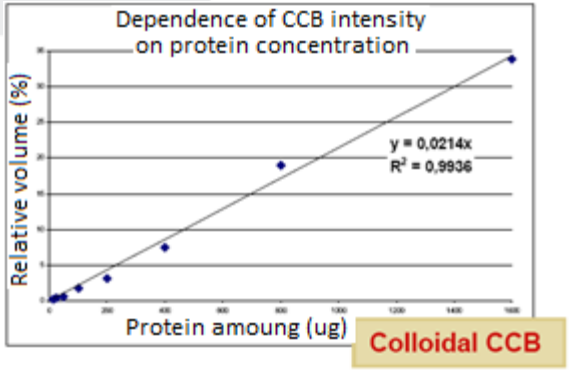
• Detection

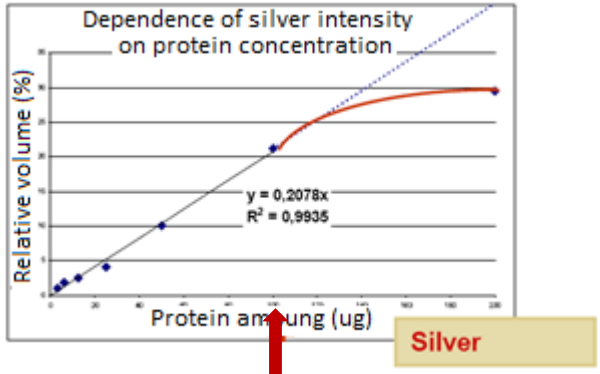
- High sensitivity
- Quantitative staining



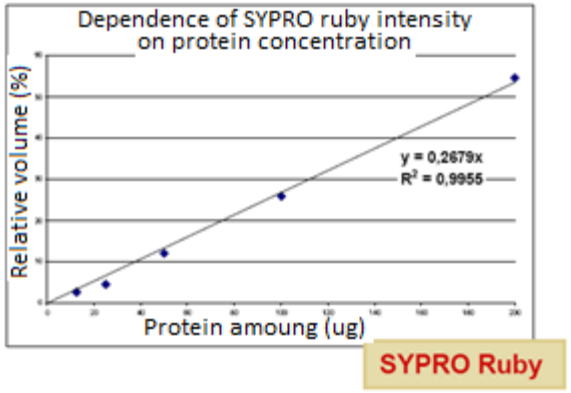
- Broad linear range of dye intensity dependence on protein amount in the gel

Dynamical range
= graph of dye intensity dependence (y axis) on protein concentration (x axis)





- end-point
- lifetime
  (e.g. quenching of fluorescent dyes!)
- Compatibility with following procedures
  (e.g. silver - glutaraldehyde!)



Silver staining
– limited linearity - only upto 100 ng of protein.

- Detection → Kind of detection

Labelling before analysis (DIGE – CyDye, radioactive labelling)

Staining after analysis

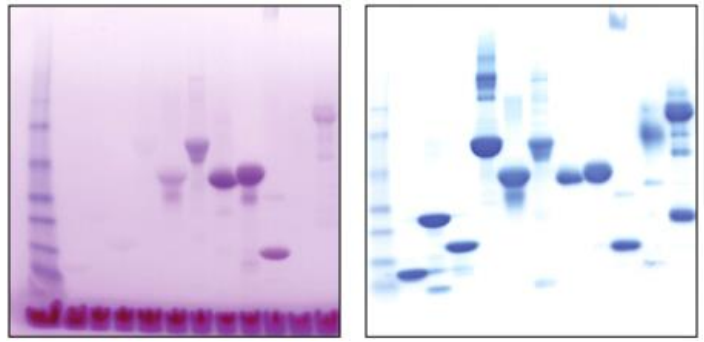
Unspecific staining: all proteins

- Visible staining: Coomassie brilliant blue (R250, G250), silver (acid x ammoniacal variant)
- Fluorescent staining: Sypro Ruby (Ex/Em = 280, 450/610 nm), Lucy (Ex/Em = 506/520 nm), Flamingo Pink (Ex/Em = 512/535 nm), Oriole (Ex/Em = 270/604 nm), Krypton (Ex/Em = 520/580), Deep Purple (Ex/Em = 365, 520/610 nm), Lumitein (Ex/Em = 280, 450/610 nm)

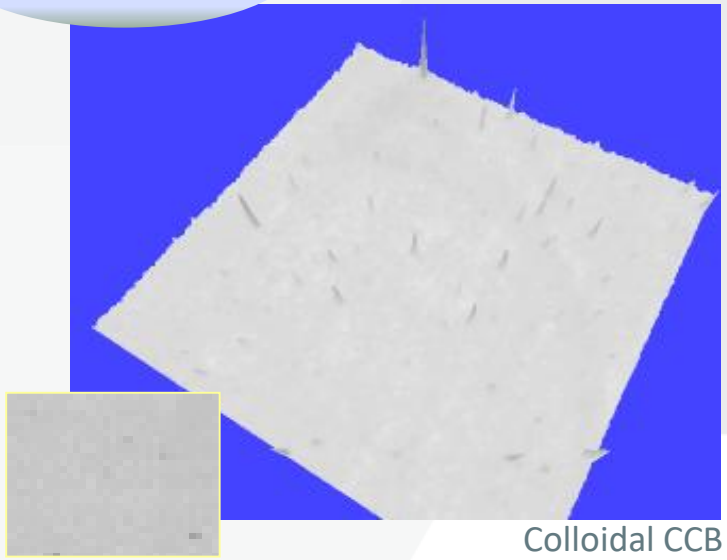
Specific staining: post-translational modifications (PTM)

- phosphorylation: Pro-Q Diamond (pSer, pThr, pTyr), Pierce phosphoprotein staining kit (pSer, pThr)

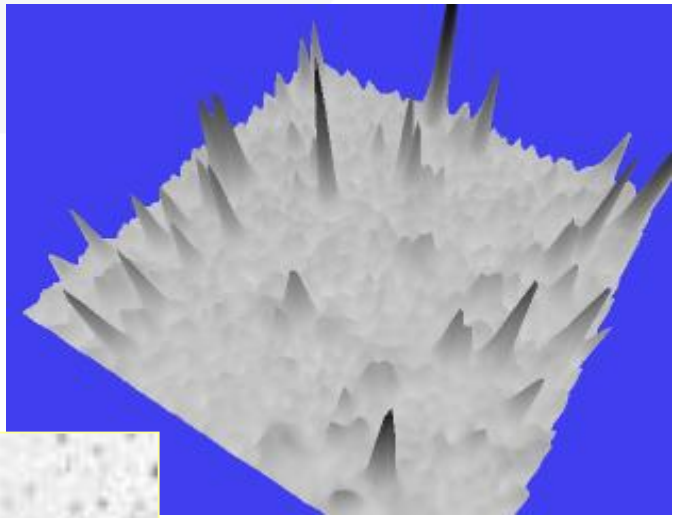
  glycosylation: Pro-Q Emerald, Pierce glycoprotein staining kit
- Radioactive labelling



Background – 3D view



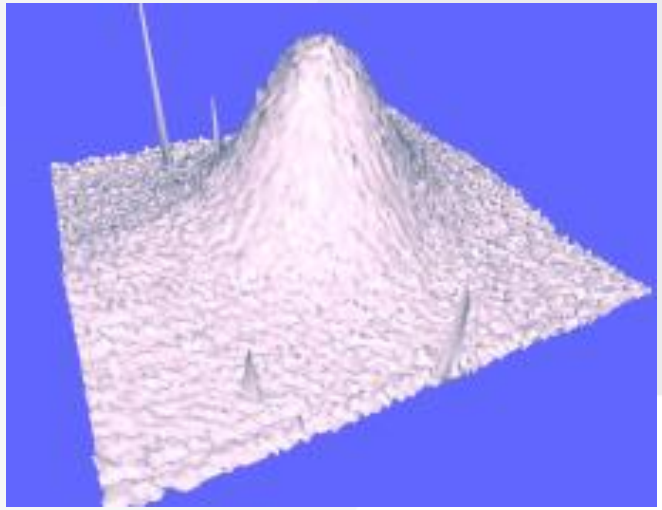
Colloidal CCB



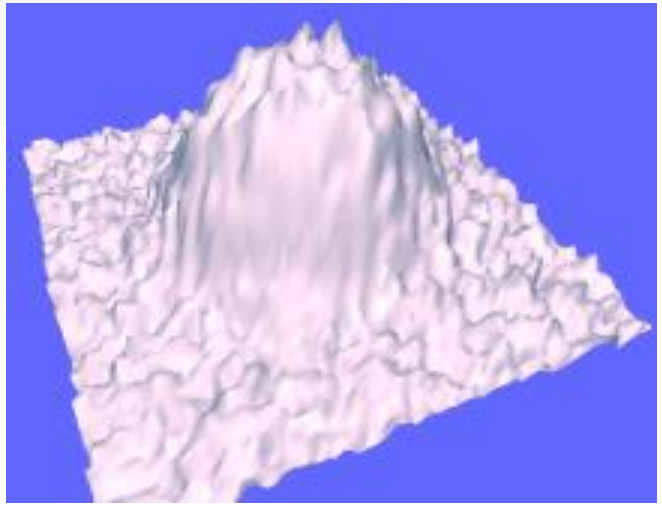
SYPRO Ruby

Silver

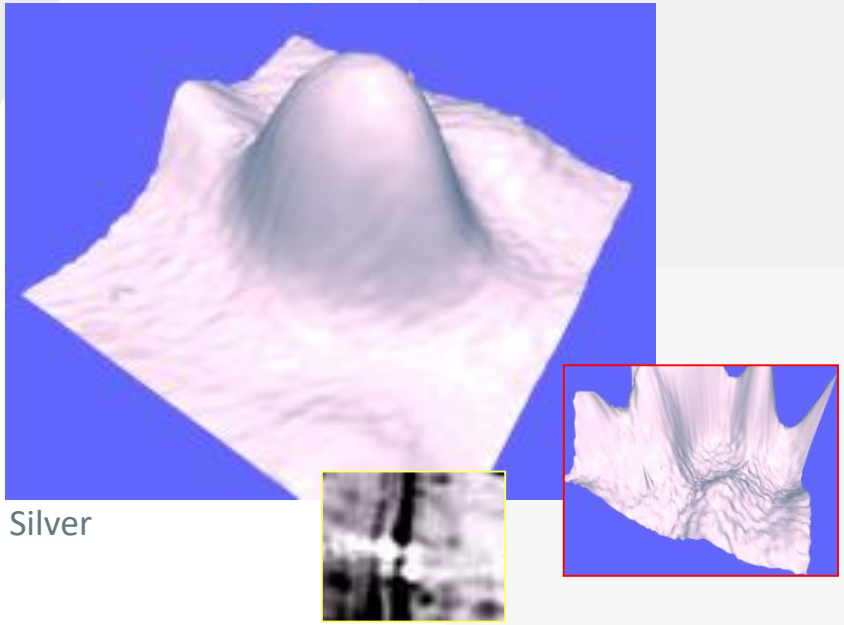
Protein spot – 3D view



Colloidal CCB



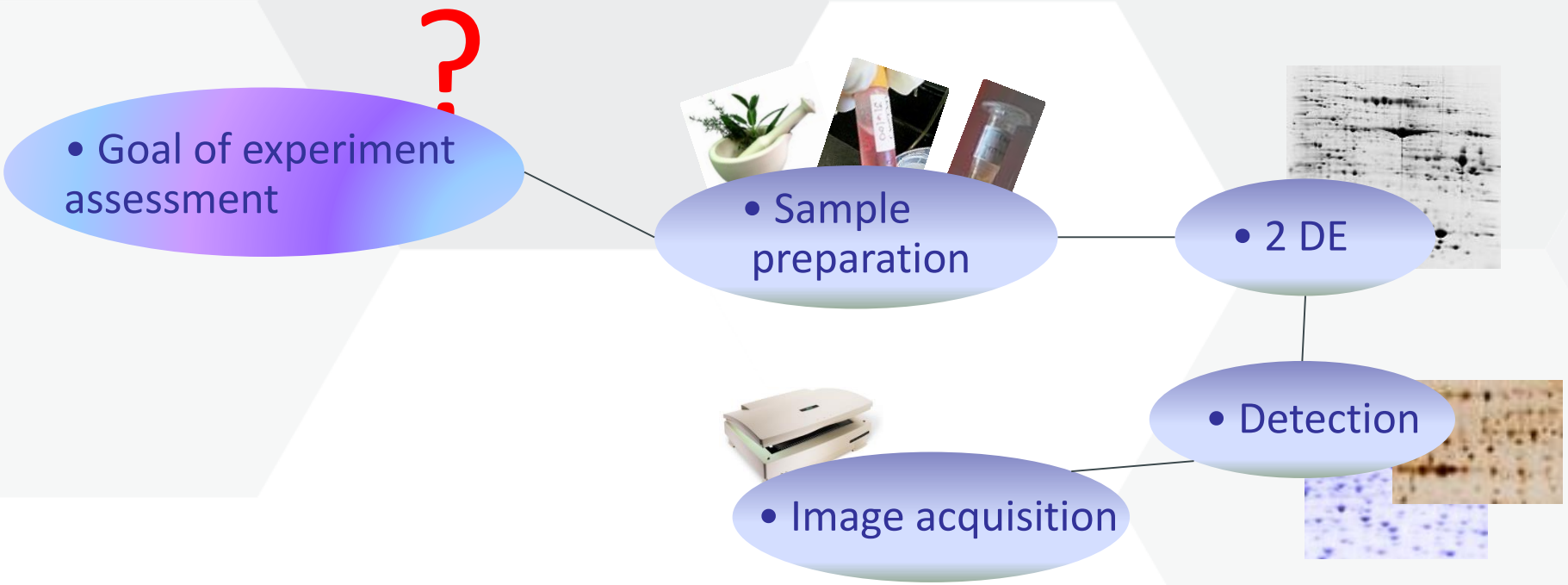
SYPRO Ruby



Silver

During image analysis we are working with density of the dye.

Experiment


?
• Goal of experiment assessment
• Sample preparation
• 2 DE
• Detection
• Image acquisition

Signals from biological samples are converted into the digital data in black and white

- TIFF format, high resolution

• Image acquisition

Instrumentation for image acquisition

Instrument choice according to type of detection used

- Visible stains : densitometers

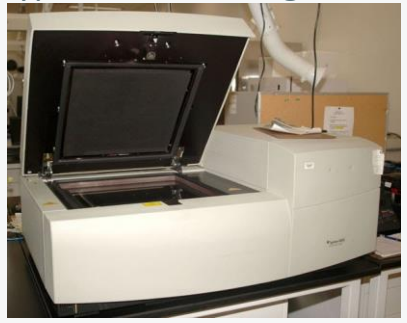
Molecular Imager GS-800

Image Scanner III

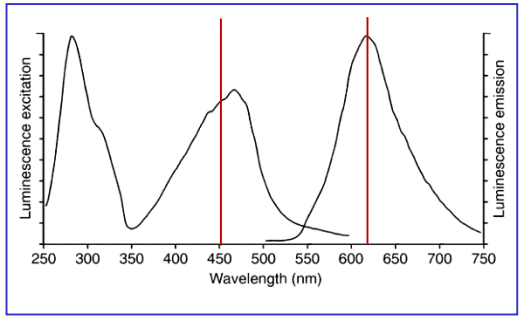
- Fluorescent stains: fluorescence scanners, cameras

  Ex/Em spectrum has to correlate with Ex/Em characteristics of the instrument

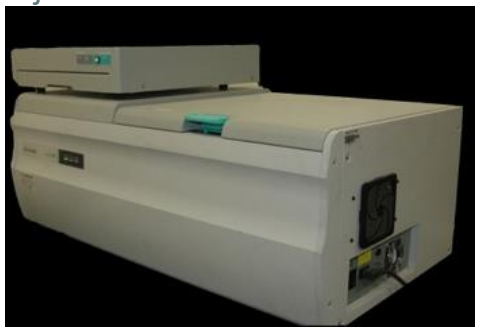
Typhoon 9200 Imager



S. Ruby: Ex/Em: 280, 450/610 nm



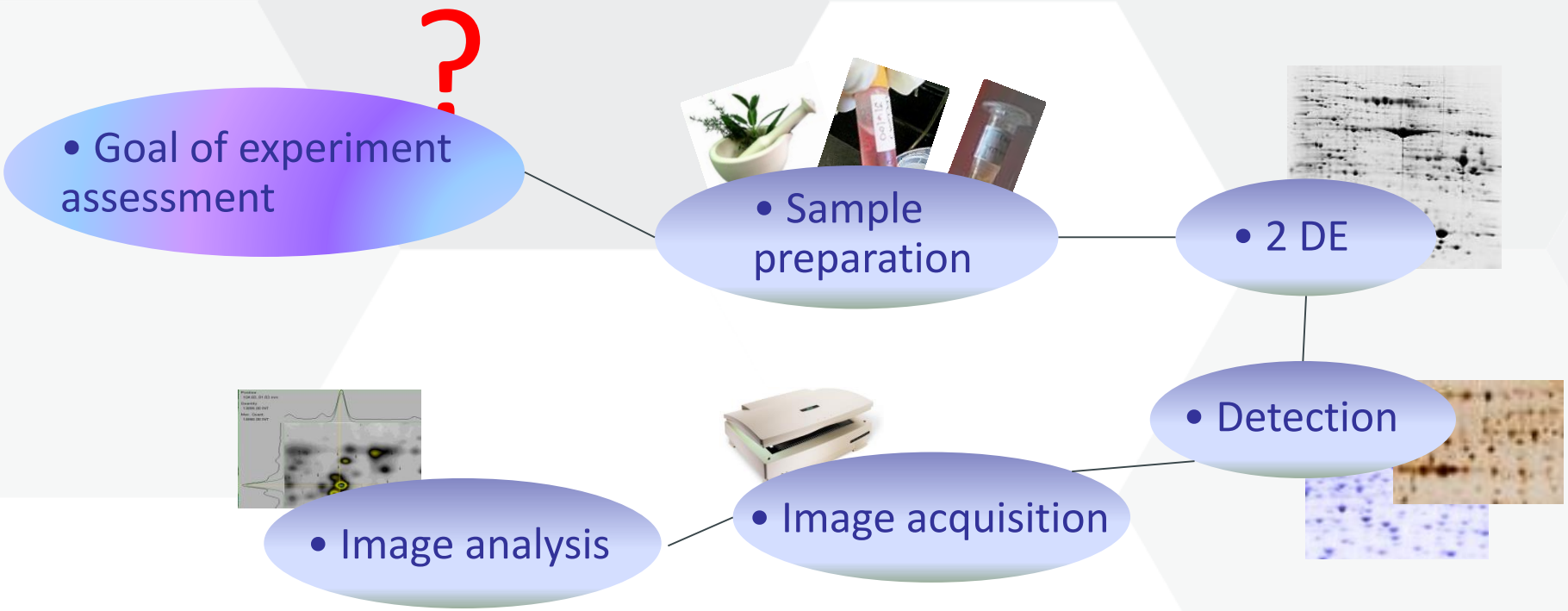
Fuji FLA-3000



PharosFX™ and PharosFX Plus Systems

CEITEC

Experiment


?
• Goal of experiment assessment
• Sample preparation
• 2 DE
• Detection
• Image acquisition
• Image analysis

• Image analysis

•Human eye distinguishes

500 shades of grey
10 million colours

Visible light only at **wave lenght of 380–760 nm**

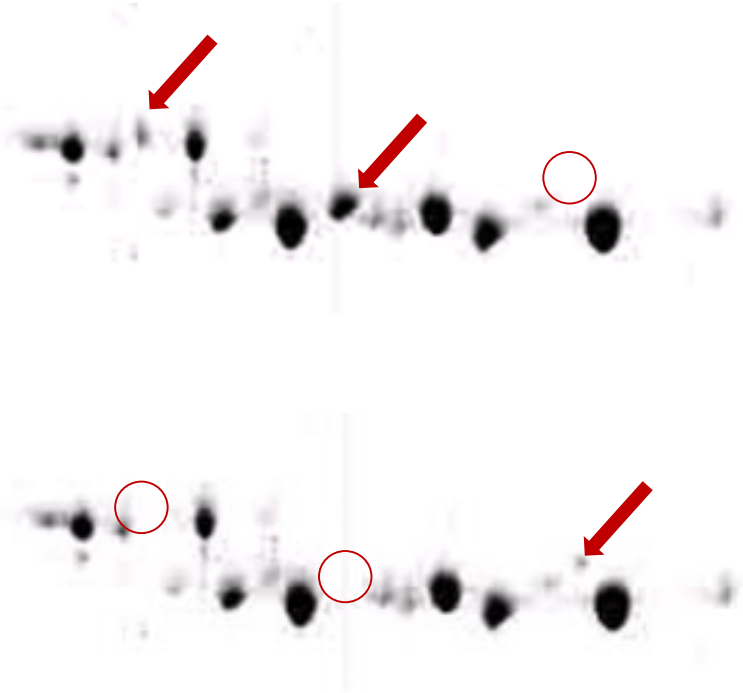
Almost ½ **of human brain participites on sight control**.

Analysis using a specialized SW

- Comparison and evaluation of 2D gels (visual evaluation of 2D gels is not possible)

- Image analysis → Analysis using a specialized SW

Qualitative evaluation



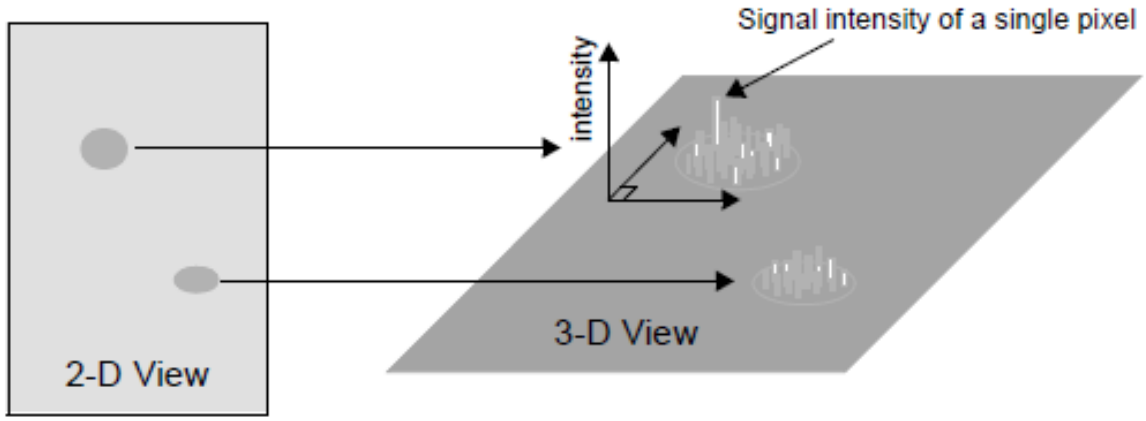
Quantitative evaluation / Statistical analysis

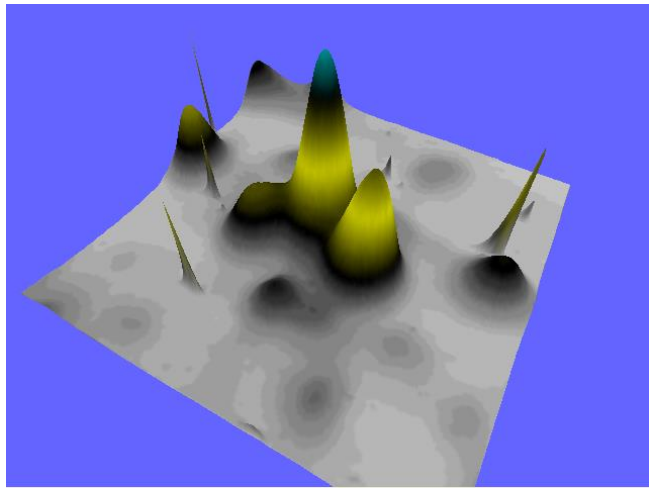
- **Spot quantity**

= total intensity of a defined spot

(for evaluation gaussian visualization is used)

- corresponds with protein amount in a particular spot





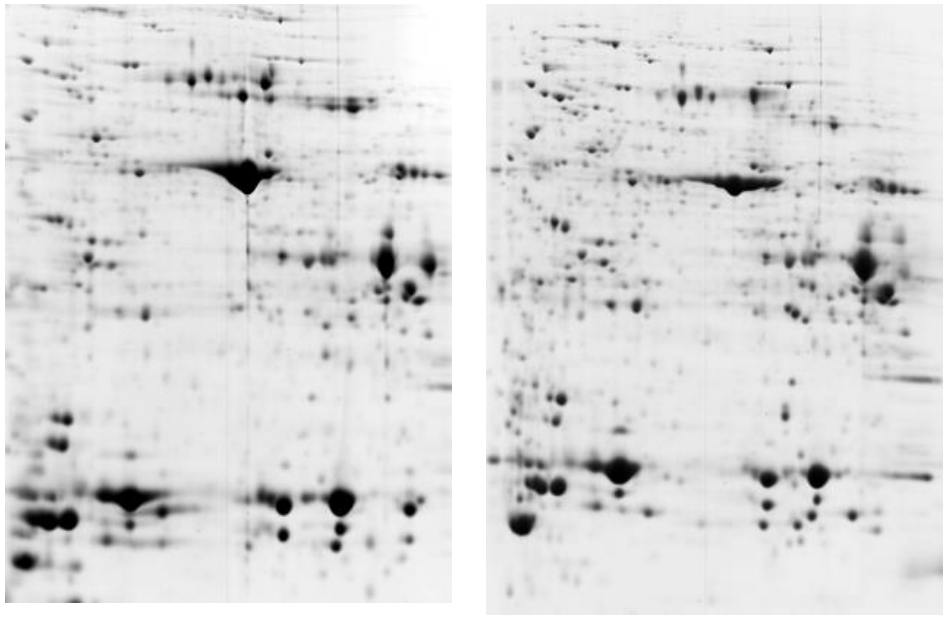
• Image analysis → Strategy according to defined goal

- treated sample x control
- time-dependent treatment

The quality of image analysis corresponds to the quality of protein separation.

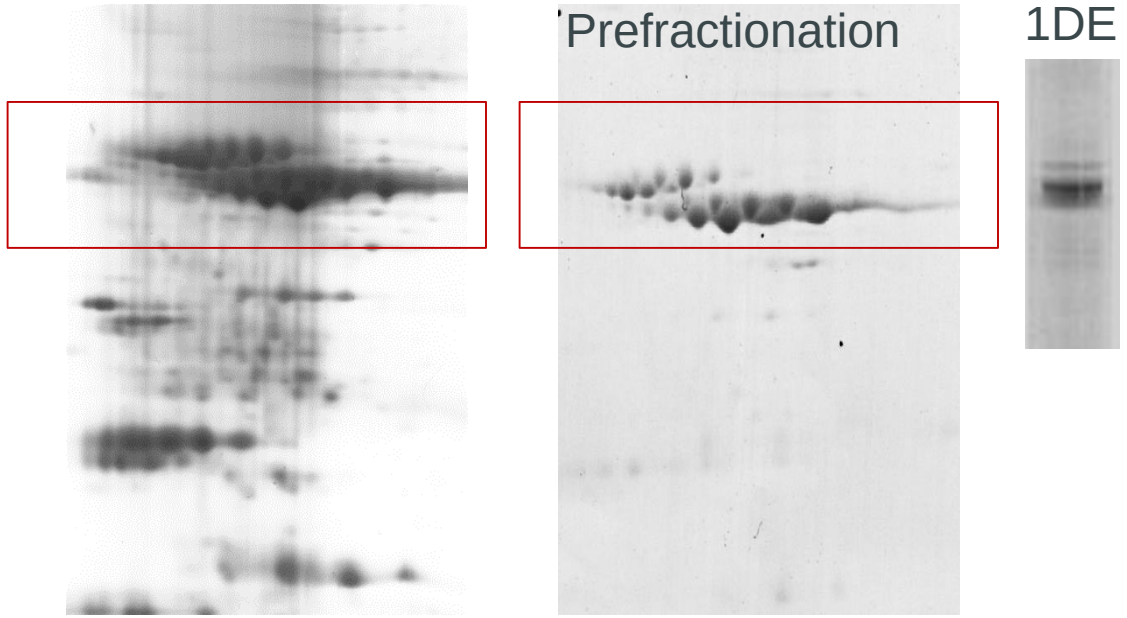
- Selection of spots which significantly differ based on certain design of experiment

– detection up- and down-regulated proteins



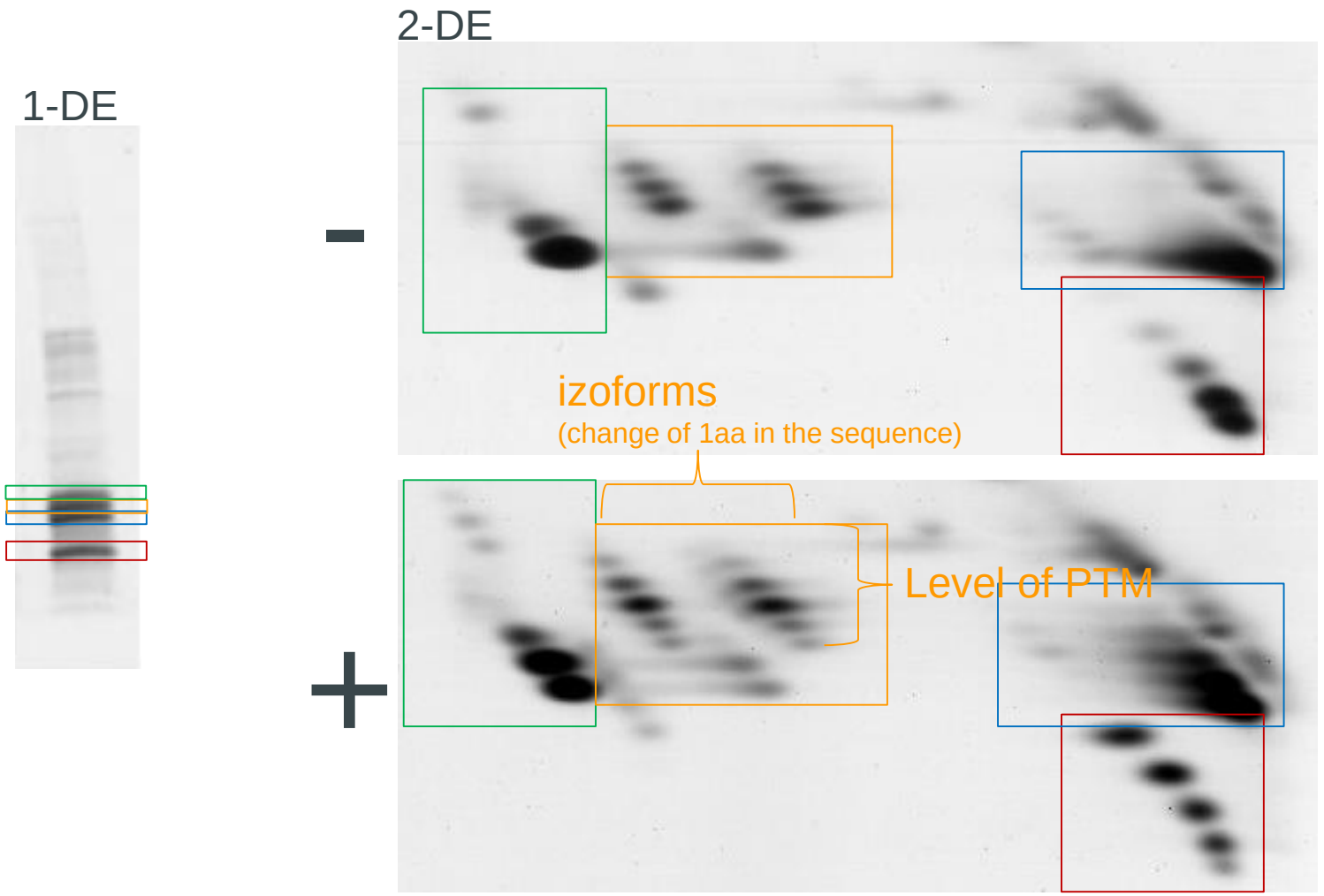
- Selection of limited number of significant spots
  - Protein isoforms
  - Post-translational modifications

– quantitative changes in the profile of certain spots



- Image analysis

Strategy according to defined goal



Evaluation using PDQuest

Image acquisition

↓

Image editing – size, orientation

↓

Spot identification

↓

Matching

↓

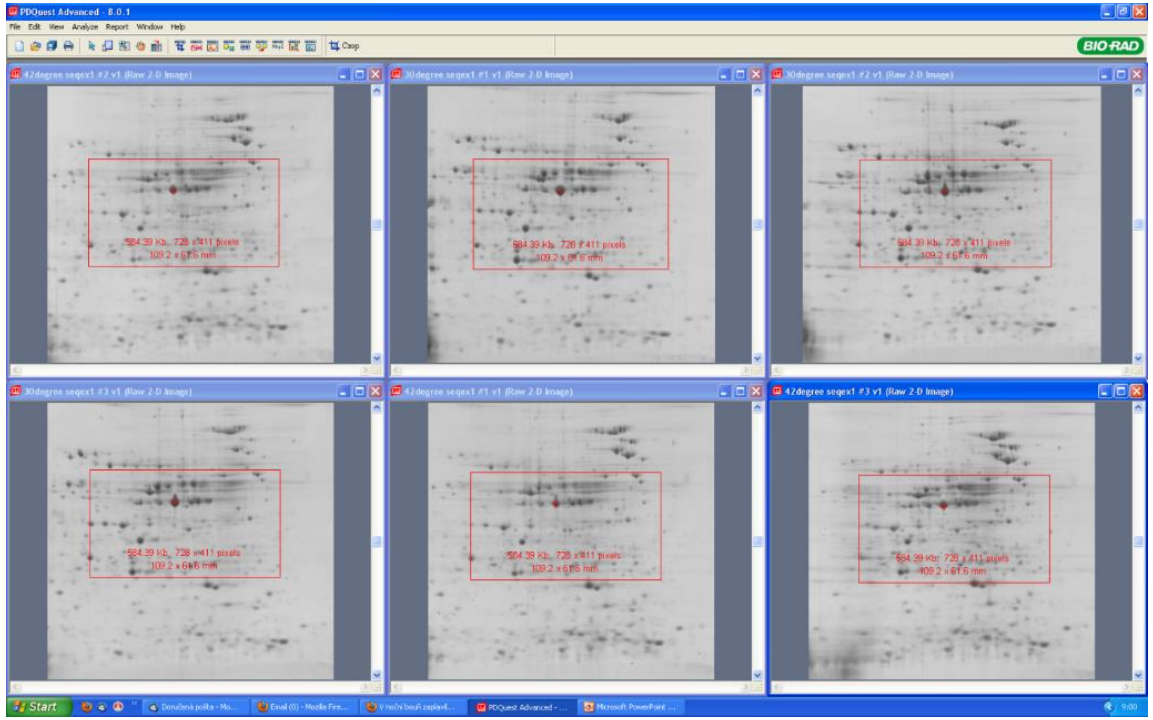
Normalization

↓

Data analysis

↓

Report



Evaluation using PDQuest

Image acquisition

↓

Image editing – size, orientation

↓

Spot identification

↓

Matching

↓

Normalization

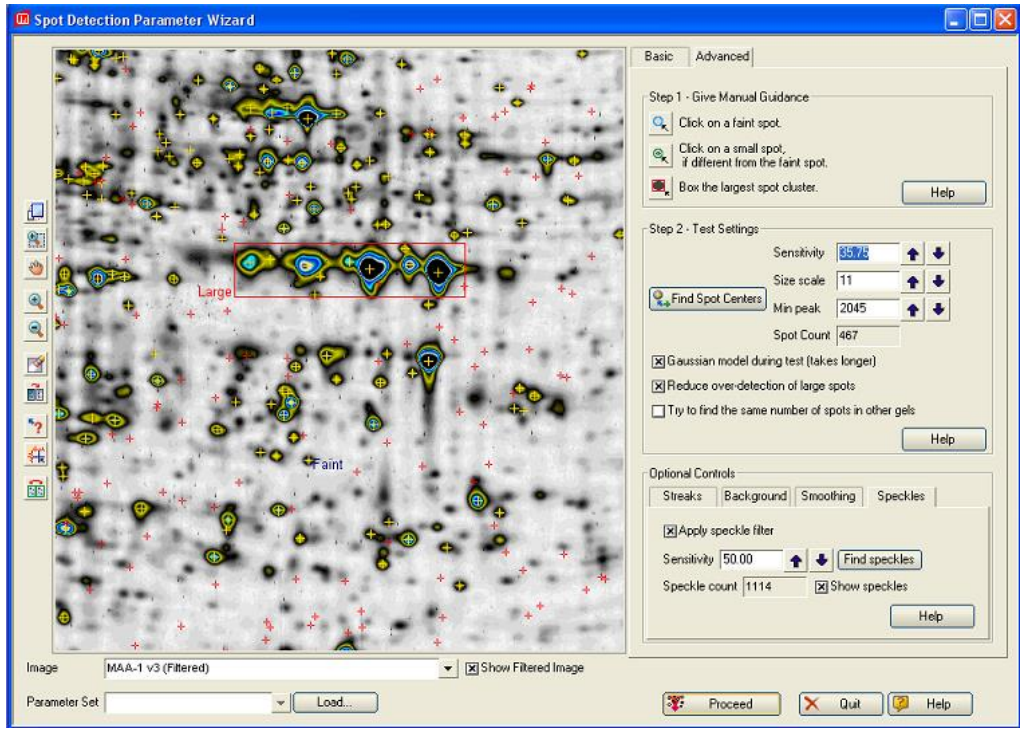
↓

Data analysis

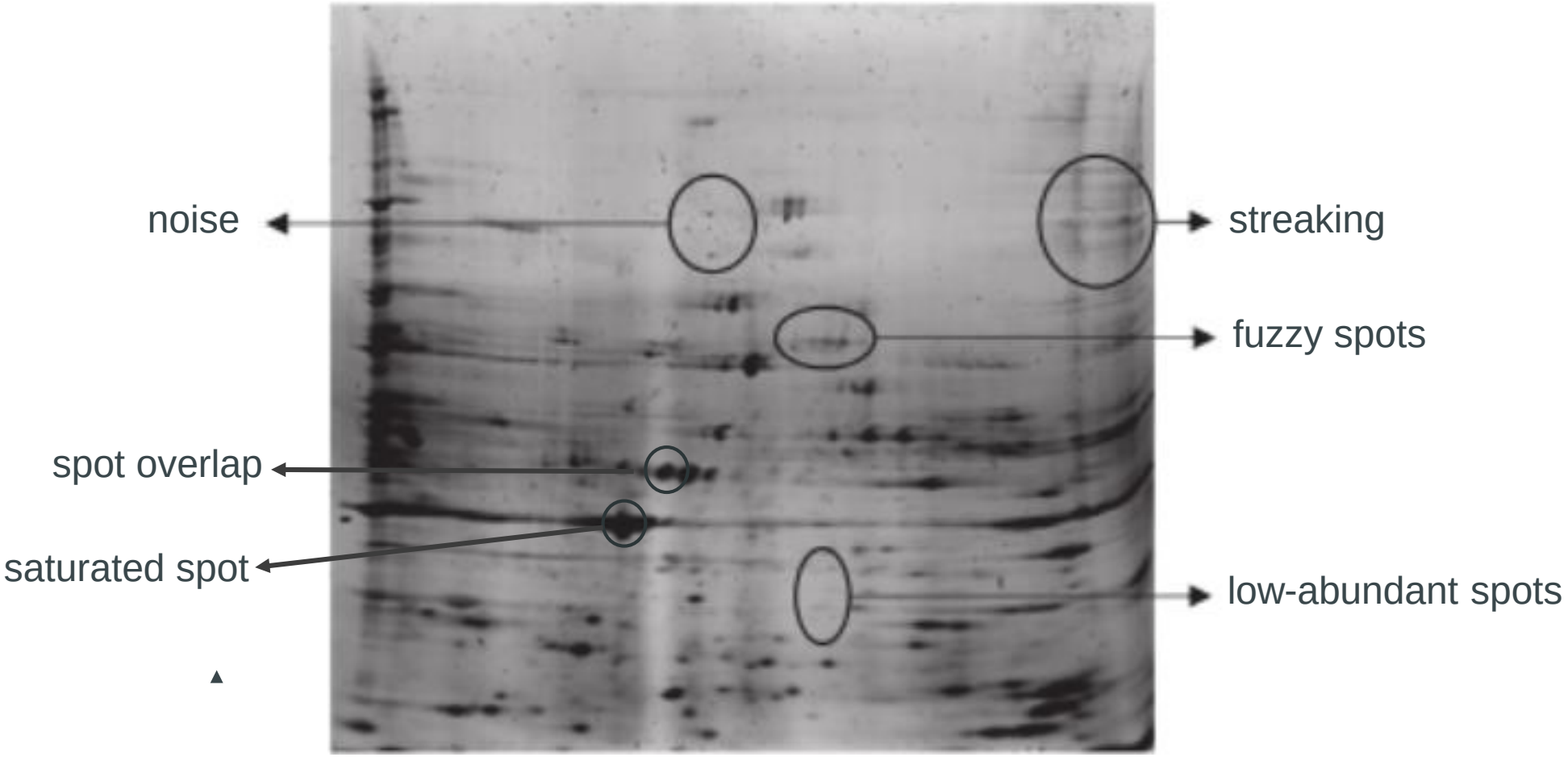
↓

Report

- Spot detection wizard
  – to select the parameters for detecting spots and background filtration in gel scans
- Different gels – different parameters needed

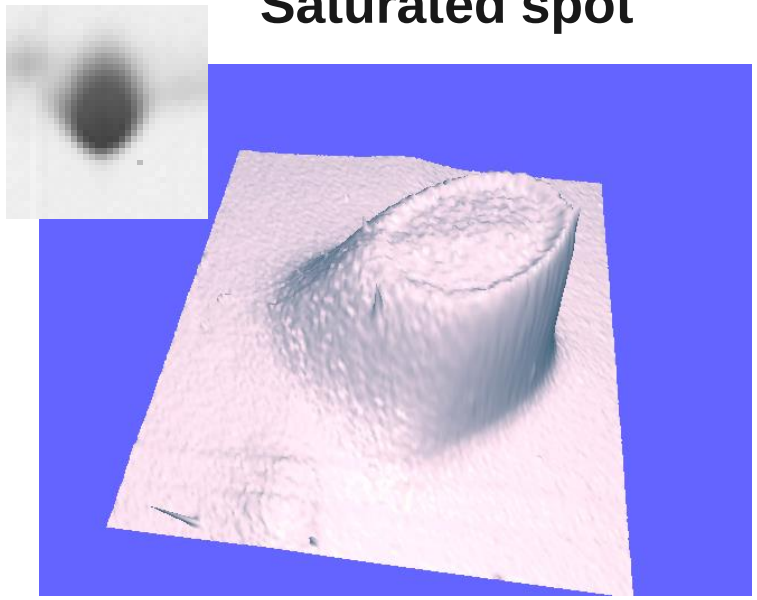


- Image analysis → Common anomalies present in 2D image

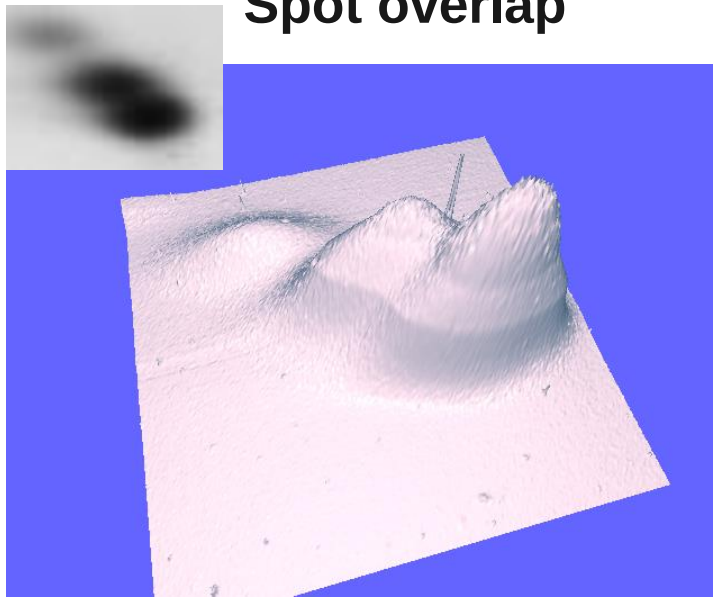


Goez et al. 2018 (modified)

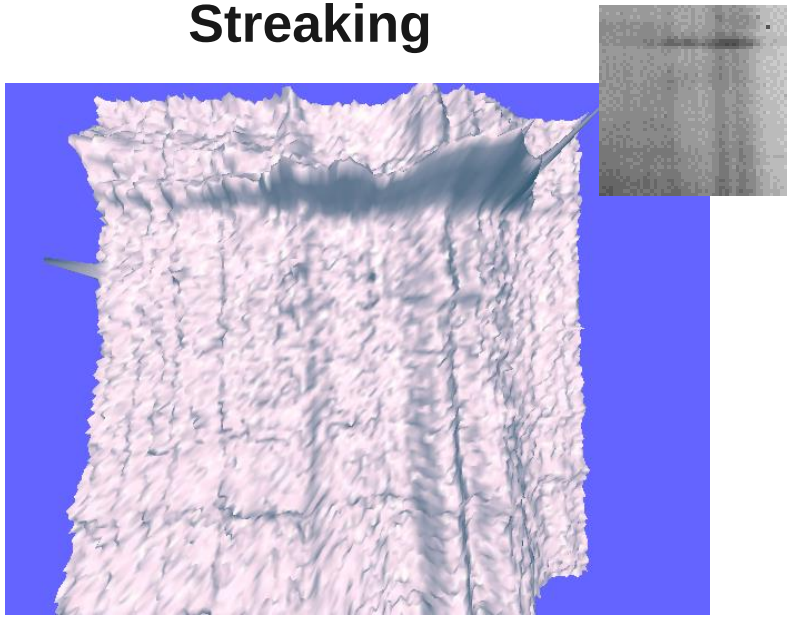
Saturated spot



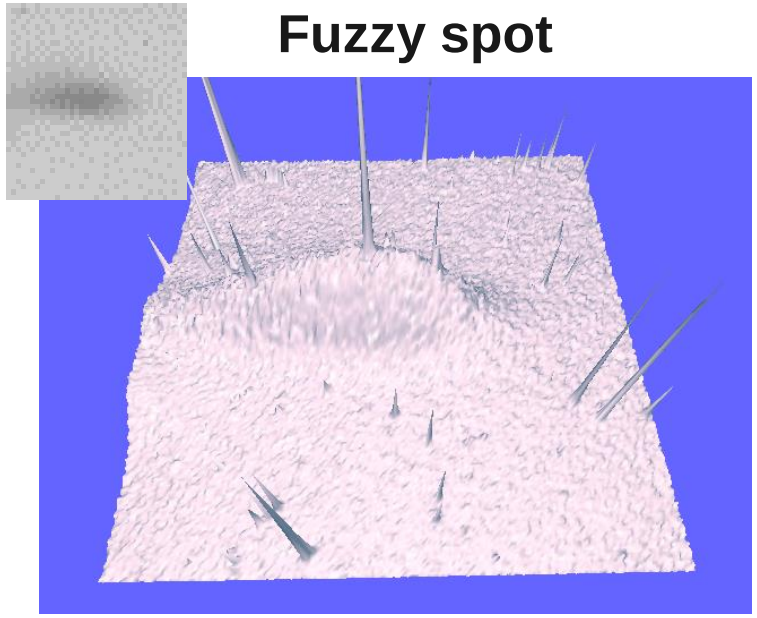
Spot overlap



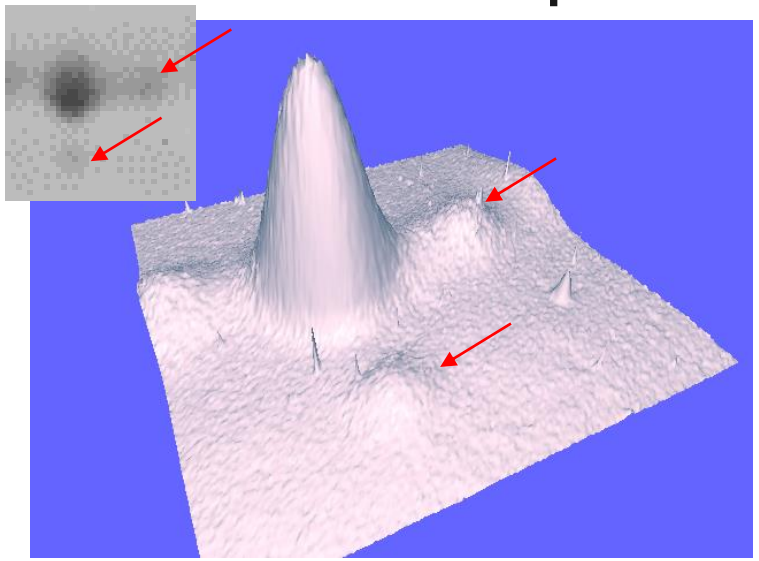
Streaking



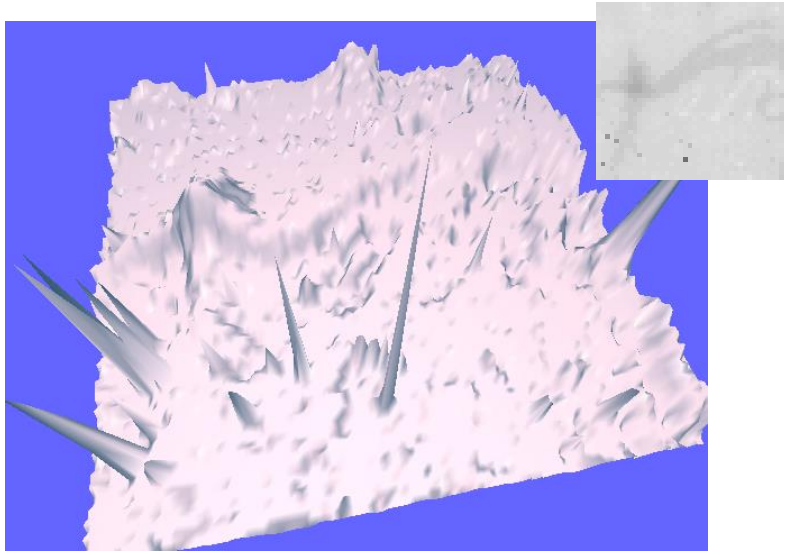
Fuzzy spot



Low-abundant spot



Noise

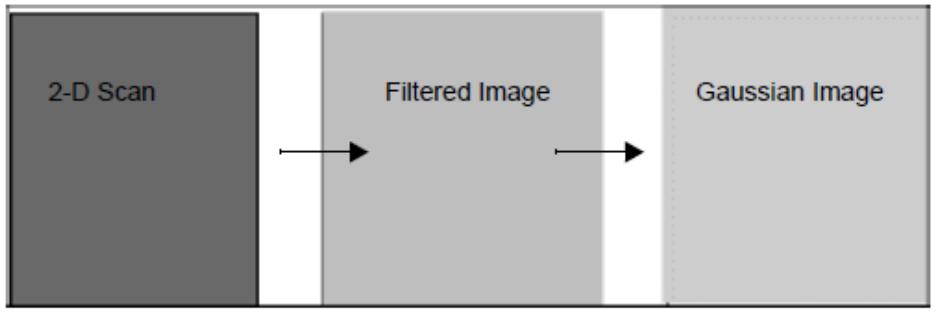


Evaluation using PDQuest

Spot detection and background filtration

Local changes in the background based on the intensity of present protein spots (higher intense spots ~ higher background)

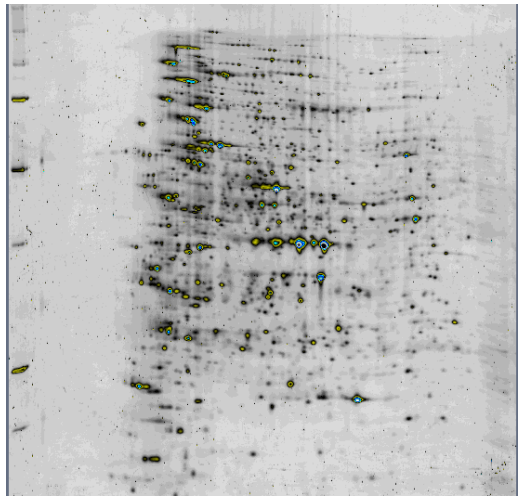
⟶ detection errors.



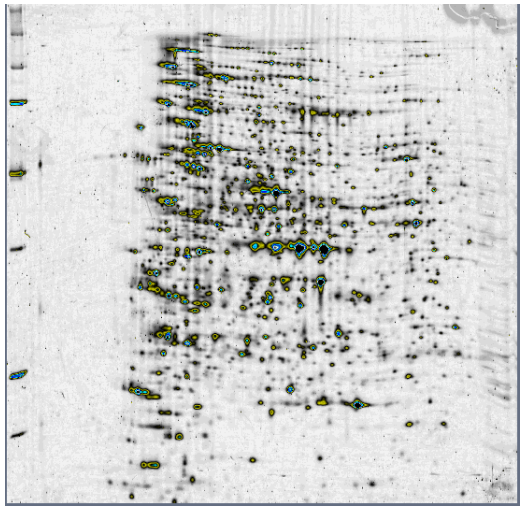
- **Scanset**

= set of images originating from a single gel (3 visualization of each gel)

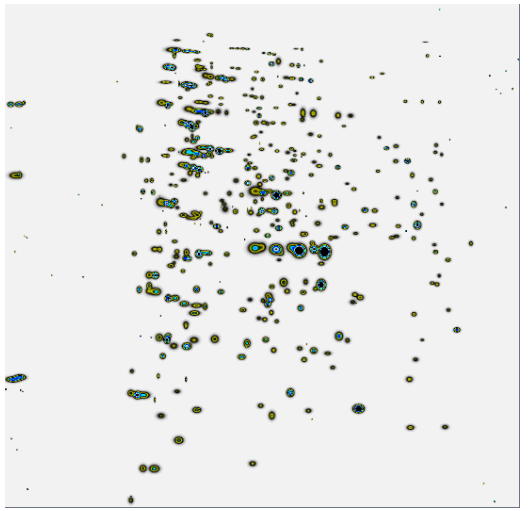
Raw 2D image



Filtered



Gaussian

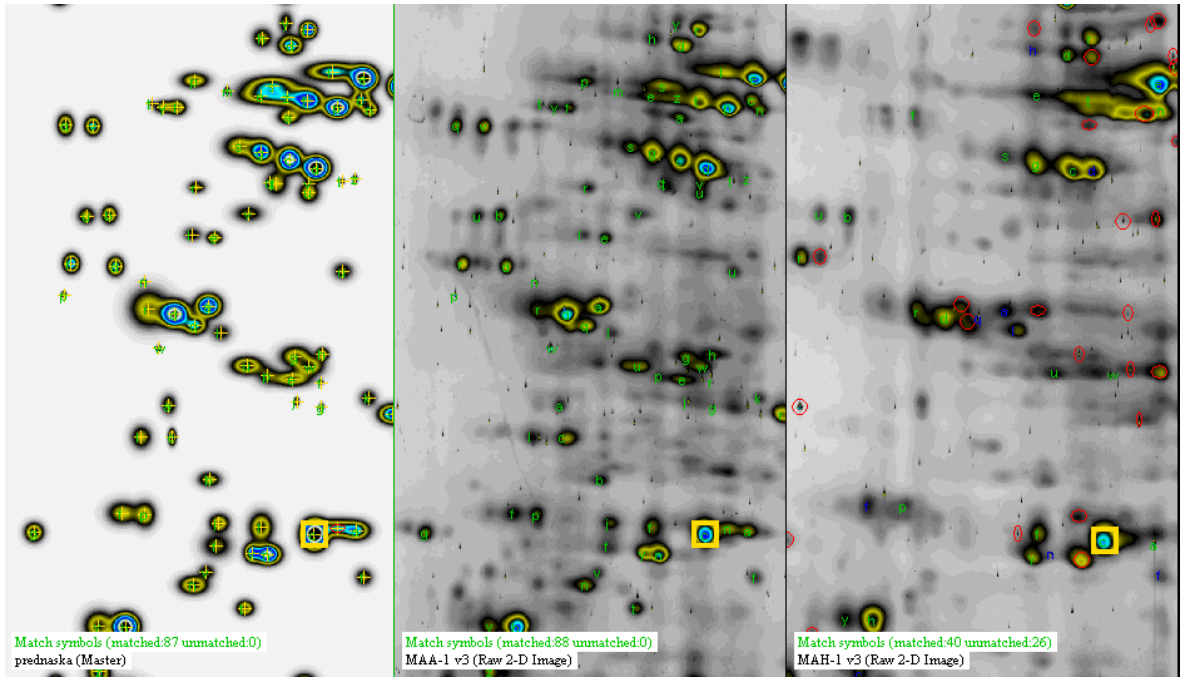


Evaluation using PDQuest

• Basic problem of image analysis: the differences in the spot position among gels
– the necessity to perform an alignment

Image acquisition
↓
Image editing – size, orientation
↓
Spot identification
↓
Matching
↓
Normalization
↓
Data analysis
↓
Report

•Matchset = set of gels which are compared among each other within a single experiment
•
• Master gel = artificial gel; involves spots from all compared gels



Evaluation using PDQuest

Image acquisition
↓
Image editing – size, orientation
↓
Spot identification
↓
Matching
↓
Normalization
↓
Data analysis
↓
Report

- Normalizace = compensation of variantion in spot size and intensity among gels that is not due to differential protein expression
- condition for appropriate spot quantity comparison

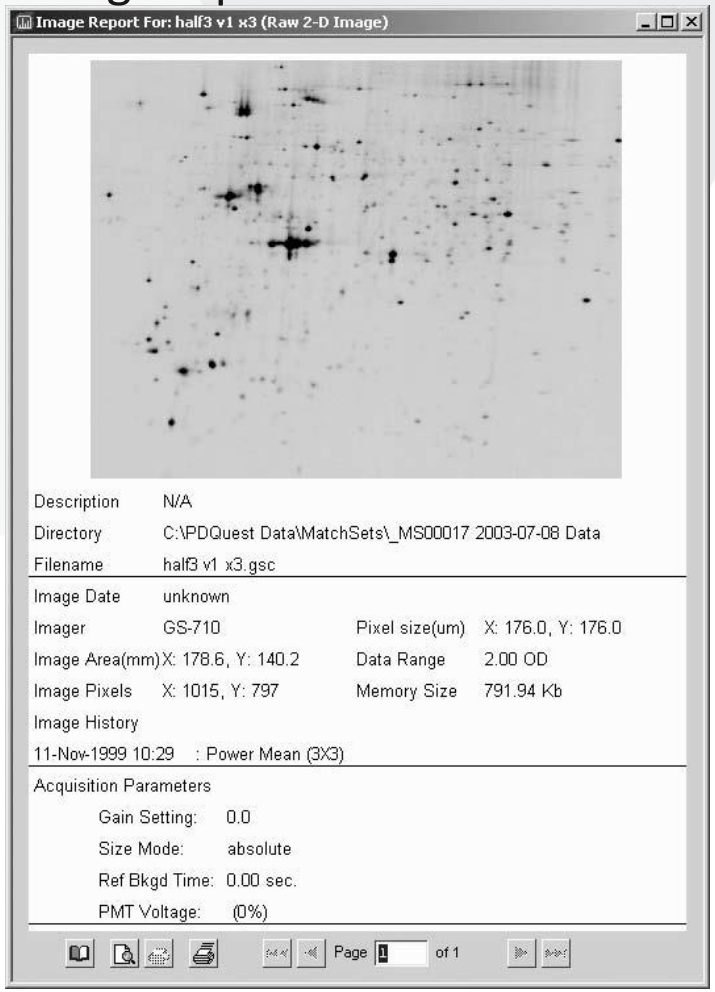
- Variance caused by different factors:
- - pipetting errors during sample prep
- - handling errors resulting in sample loss during sample prep
- - sample loss during transfer from strip to gel
- - inconsistent staining among gels
- - inconsistent detection energy sources among gels during image acquisition
- - .....

- Normalization factor (according to selected method)
  - Total quantity in analysis set
  - Total quantity in valid spots
  - Total density in gel image
  - Specified value
  - Mean of log ratios
  - Local regresion model

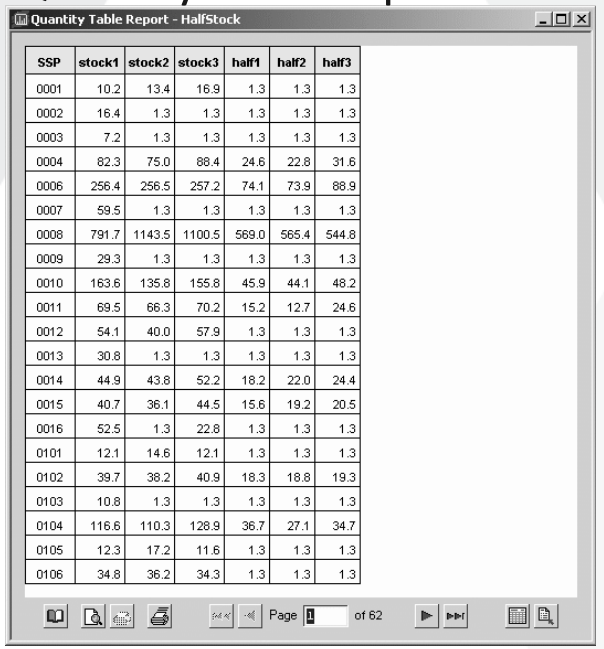
Evaluation using PDQuest

• Report

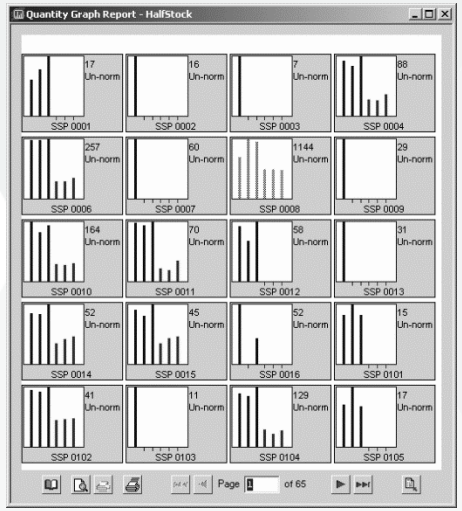
Image report



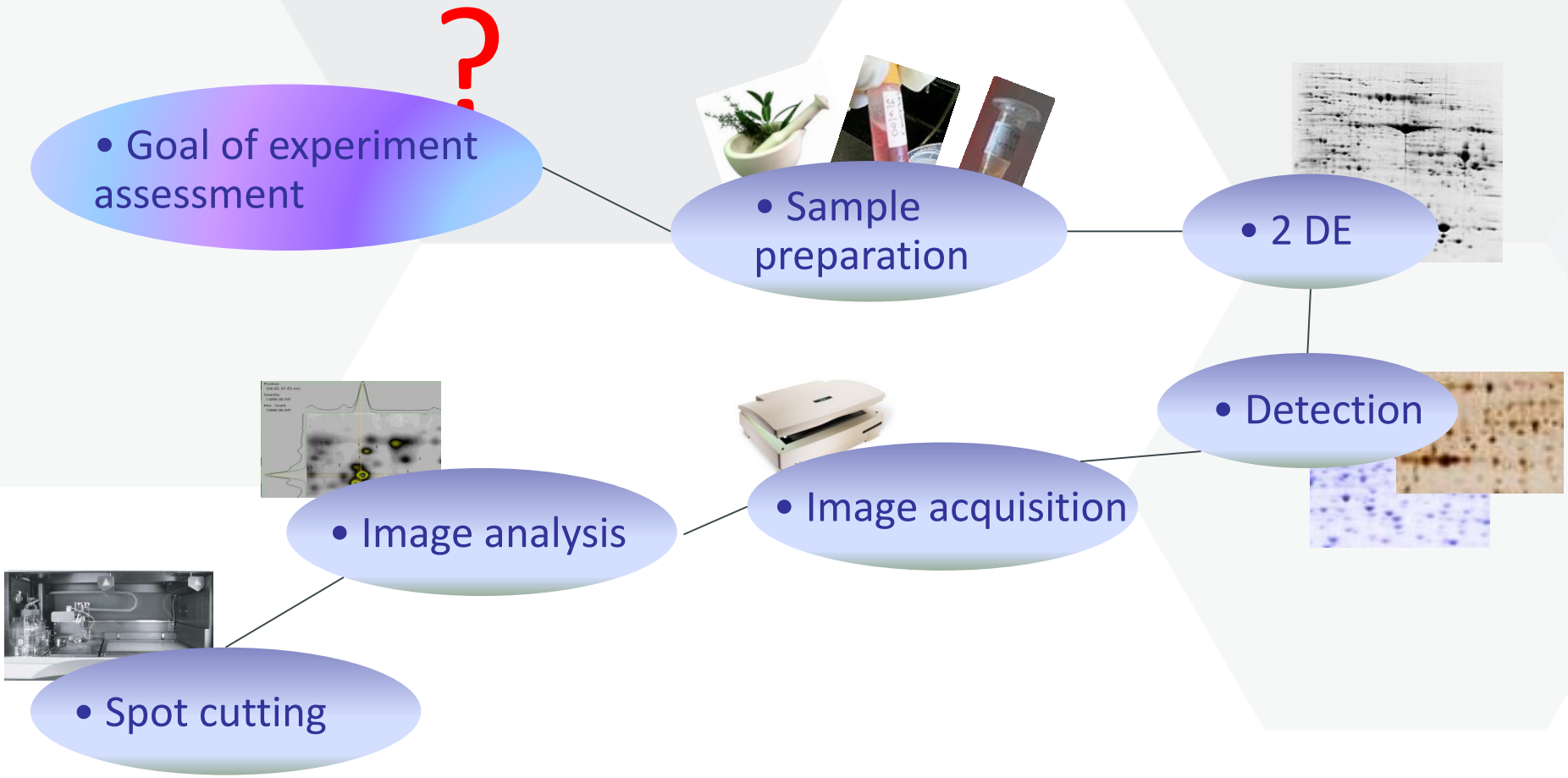
Quantity Table report



Quantity Graph report



Experiment


?
• Goal of experiment assessment
• Sample preparation
• 2 DE
• Detection
• Image acquisition
• Image analysis
• Spot cutting

- Spot cutting

Manually (scalpel, spot picker)

- Visible stains
- Fluorescent stains
  - transiluminator

OneTouch Plus spot picker

UV-transilluminator

(Ex: 302, 365 nm)

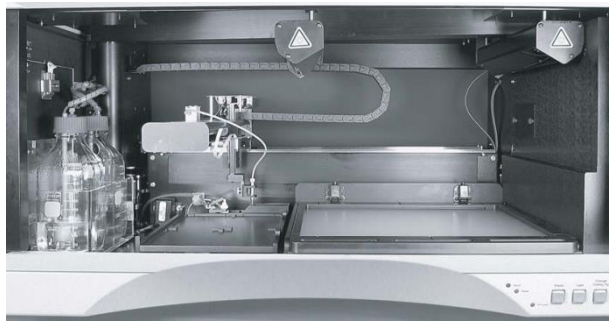
Dark Reader

(Ex: 490 nm)

Automation

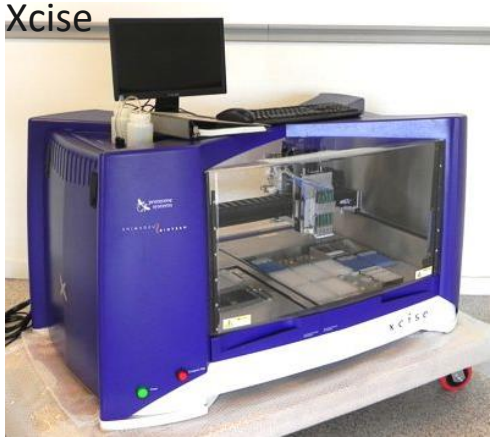
- Spot cutter

Exquest



Ettan Spot picker

Xcise



• Goal of experiment assessment
• Sample preparation
• 2 DE
• Detection
• Image acquisition
• Image analysis
• Spot cutting
• Digestion
• Identification
• Goal of experiment

2-DE

- The best method of protein visualization in the form of protein spot which might be characterized its abundance, localization, presence / absence.

- The most offen anomalies influencing image analysis:
  vertical and horizontal streaking, fuzzy spots, saturated spots, low-abundant spots, spot ovelap, noise.

- Laborious, time consuming method, limited reproducibility, limited resolution (1 spot ≠ 1 protein!).

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Thank you for your attention.

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