Chapter 33
Antibody Microarrays for Expression Analysis

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33.1 Introduction

In recent years, antibody microarrays have developed into an important tool for proteomics. As a multiplexing technique, they facilitate the highly parallel detection of hundreds of different analytes from very small sample volumes of only few microliters. This is combined with a high sensitivity in the picomolar to femtomolar range, which is similar to the sensitivity of ELISA, the gold standard for protein quantification. In order to obtain such sensitivities in a robust and reproducible manner for sets of several hundreds of analytes simultaneously, it is essential to optimise the experimental layout, sample handling, labelling and incubation as well as data processing steps. Here, we present our current antibody microarray protocols for multiplexed expression profiling studies, which permit the analysis of the abundance of more than 800 proteins in plasma, urine and tissue samples.

Antibody microarray experiments comprise four major steps: array production (Sect. 33.3.1), sample preparation (Sect. 33.3.2), incubation (Sect. 33.3.3), and finally image acquisition and data analysis (Sect. 33.3.4).

For array production (Fig. 33.1a), different antibodies are immobilised covalently at distinct locations on a planar surface. Subsequently, the surface is blocked in order to minimise unspecific protein adsorption. Protein samples are extracted from different sources such as plasma, serum, urine or tissue. The analysis procedure depends on the detection strategy. There are three major options: the classical sandwich strategy, the hapten strategy, and the fluorescent-dye strategy. For the classical sandwich approach, no modification of the samples is needed. The antigens are captured by the respective antibodies on the surface (Fig. 33.1b) and are recognised in a separate incubation step by a second set of antigen-specific

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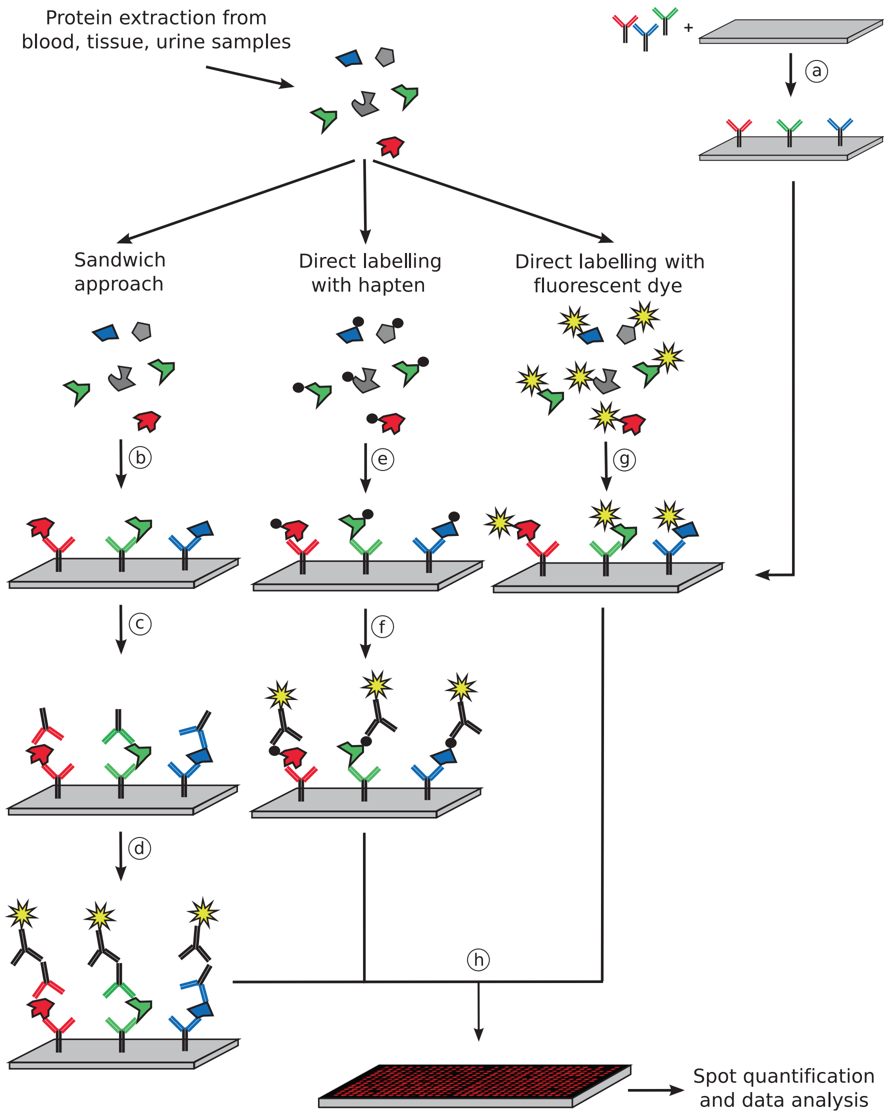

Fig. 33.1 Schematic representation of an antibody microarray experiment

antibodies (Fig. 33.1c). For detection, the arrays are incubated in a third step with a fluorescently labelled antibody (Fig. 33.1d), which recognises a common feature of the second binder set.

In the other detection strategies, samples are labelled with either a fluorescent dye (Fig. 33.1g) or a hapten such as biotin or DNP (Fig. 33.1e). Labelling with a fluorescent dye facilitates a direct readout, whereas hapten labelling requires an additional incubation step with a specific antibody (Fig. 33.1f). Fluorescence signals

Table 33.1 Comparison of different labelling and detection strategies

	Labelling and detection strategy		
	Fluorescent Dye	Hapten	Sandwich
Direct labelling of protein samples	+	+	–
Direct detection possible	+	–	–
Easy scale-up of analyte number for complex analyses	+ +	+ +	– –
Sensitivity for small number of analytes[a,b]	+	+	+ +
Sensitivity for large number of analytes[a]	+	+ +	+
Signal amplification method such as rolling circle amplification[c] possible	–	+	+
Simplicity of use/robustness	+ +	+	–
Number of incubation and washing steps	1	2	3
Number of antibodies per analyte	1	1	2
Costs per analyte determined by	Antibody	Antibody	Pair of antibodies
Costs per sample determined by[d]	Fluorescent dyes	Haptens	Second set of antibodies
Competitive two colour assays and thus reduced technical variability possible	+	+	–

[a]Kusnezow et al. (2007)
[b]Wingren et al. (2007)
[c]Schweitzer et al. (2000)
[d]Additionally to fix costs such as surfaces, chemicals

are recorded using a microarray scanner (Fig. 33.1h) and subsequently converted into signal intensities using a software for spot recognition and quantification.

The choice of the detection strategy largely influences sensitivity, the degree of multiplexity and the robustness of the assay. The optimal process depends on factors such as the number of analytes, the number and kind of samples to be analysed as well as the sensitivity needed for the respective application. The particular properties, advantages and disadvantages of the different strategies are summarised in Table 33.1.

For choosing the appropriate labelling, the experimental design should also be considered. Using the sandwich strategy, each sample needs to be incubated separately on one array. In order to compare two different samples (e.g. plasma samples from normal and diseased patients), signals derived from two different arrays have to be compared to each other. The same layout is possible for the hapten and fluorescent-dye strategies using a one-colour assay (Fig. 33.2a). However, these detection strategies also provide the possibility to perform a two-colour assay as commonly done with cDNA arrays for good reason. Samples are labelled with different fluorescent dyes or haptens and incubated in a competitive manner on the same array. This facilitates a direct comparison of the signals and thereby minimises possible variation effects. For small numbers of samples, such dual-colour comparisons can be performed comprehensively, e.g. all samples versus all samples (saturated design). In practice, the number of comparisons is limited by sample amount or costs per comparison. A pairwise comparison, e.g. between healthy and diseased tissues of the same patient (Fig. 33.2b) is most cost-effective,

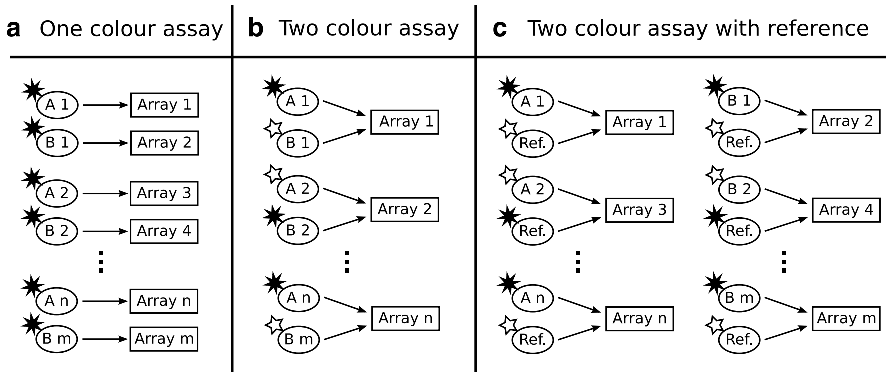

Fig. 33.2 Experimental layouts for microarray experiments. A scheme is shown for two different biological factors (A, B, e.g. A = normal, B = cancer) with biological replicates (1, 2, n, m). For two-colour assays, a dye switch should be integrated to estimate dye specific effects

but the acquired data do not capture any difference among the patients that is unrelated to the disease. This is particularly limiting for studying co-factors such as gender, age or smoking behaviour, whose impact on the disease should be complemented with (and well distinguished from) the differences they relate to among healthy tissues. Complete information in this respect can be obtained cost-effectively by incubating each sample on a different array competitively with a reference sample, which is identical for all arrays (reference design, Fig. 33.2c). Ideally, the reference sample represents a pool of all samples of the respective project, comprising all proteins present in any of the conditions under study.

In conclusion, careful planning of the experimental procedure as well as choosing of a proper detection strategy and experimental design is essential for antibody microarray experiments in accordance with the actually addressed question.

33.2 Materials

33.2.1 Laboratory Equipment

- Micro-arraying robot: several commercial models are available. Protocols have been established using the contact printers MicroGrid 2 (Genomic Solutions, Ann Arbor, USA) and SDDC-2 (ESI, Toronto, Canada) as well as the contact-free piezo spotter NP-2 (GeSiM, Großerkmannsdorf, Germany).
- Microarray scanner: several commercial models are available. The protocols described here have been established using the ScanArray 4000XL (Perkin Elmer, Waltham, USA).
- Advalytix Slidebooster (Olympus Life Science Research, Munich, Germany).
- Centrifuges for 384-well plates, microtubes and 15 mL Falcon tubes.

- Software for image segmentation and spot recognition of the signals obtained on the microarrays, e.g. GenePix Pro (Molecular Devices, Sunnyvale, USA), Mapix (Innopsys, Carbonne, France) or TIGR Spotfinder (Saeed et al. 2003).
- Software for data analysis, several open-source packages as well as commercial programmes are available. (see Note 15)

33.2.2 Materials

- Epoxy-coated slides (Nexterion E, Schott, Jena, Germany).
- Slide racks and containers (no. 2285.1, Carl Roth GmbH, Karlsruhe, Germany).
- Poly- or monoclonal antibodies, affinity-purified in PBS with a concentration of 2 mg/mL (see Note 1 and Sect. 33.3.1.1).
- Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA).
- Fluorescent dyes (Dy549-NHS, DY649-NHS, Dyomics, Jena, Germany) or haptens such as NHS-LC-LC-Biotin (Thermo Fisher Scientific, Waltham, USA) or DNP-X-SE (Invitrogen, Paisley, UK).
- Pierce Zeba Spin Desalting Columns 2 mL (Thermo Fisher Scientific, Waltham, USA).
- Homemade plexiglas incubation chambers, which have slightly larger inner dimension than the spotting area and can be reversibly attached to the slides by double-sided adhesive tape. As an alternative, LifterSlips or Gene Frames (Thermo Fisher Scientific, Waltham, USA) can be used (see Note 2).
- Complete protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). Prepare a 25× stock solution by dissolving one tablet in 2 mL of H_2O.
- Sypro Ruby protein blot stain (S4942, Sigma-Aldrich Corp., St. Louis, USA).
- Hydroxylamine 50% (w/v) in H_2O (467804, Sigma-Aldrich Corp., St. Louis, USA). *Attention: hydroxylamine is harmful and dangerous to the environment.*

33.2.3 Buffers and Stock Solutions

If not stated otherwise, deionised water from a Millipore unit was used for the preparation of buffers and stock solutions.

- 10 × PBS buffer
  - Dissolve 80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 mL H_2O.
  - Adjust pH to 7.4.
  - Adjust volume to 1 L with H_2O.
  - Autoclave for sterilisation.

- 0.1 M Sodium borate buffer (pH 9.0)
  - Dissolve 9.535 g $Na_2B_4O_7 \cdot 10\ H_2O$ in 200 mL H_2O.
  - Stir the solution until it clears.
  - Adjust the pH to 9.0 with H_3BO_3.
  - Adjust the volume to 250 mL with H_2O.
  - Autoclave for sterilisation.
- 10% Dextran stock solution (w/v)
  - Dissolve 1 g of dextran (no. 31394, Sigma-Aldrich Corp., St. Louis, USA) in 10 mL H_2O. Store at 4°C until use.
- 10% Trehalose stock solution (w/v)
  - Dissolve 1 g of trehalose in 10 mL H_2O. Store at 4°C until use.
- 1% Igepal stock solution (v/v)
  - Add 100 μL Igepal CA-630 (no. I3021, Sigma-Aldrich Corp., St. Louis, USA) to 9.9 mL H_2O. Store at 4°C until use.
- 5% Sodium azide stock solution (w/v)

  Attention: sodium azide is very toxic and dangerous to the environment.
  - Dissolve 2.5 g NaN_3 in 50 mL H_2O. Store at room temperature or at 4°C.
- 20% Tween-20 stock solution (w/v)
  - Add 20 g of viscous Tween-20 to 70 mL H_2O.
  - Stir well.
  - Adjust volume to 100 mL.
  - Filter for sterilisation.
- 20% Triton-X100 stock solution (w/v)

  Attention: Triton X-100 is irritant and dangerous to the environment.
  - Add 20 g of viscous Triton X-100 to 70 mL H_2O.
  - Heat and stir to dissolve.
  - Adjust volume to 100 mL.
  - Filter for sterilisation.
- 1 M Sodium bicarbonate buffer (pH 9.0)
  - Dissolve 4.2 g sodium bicarbonate in 50 mL H_2O.
  - Adjust to pH 9.0.
  - Autoclave for sterilisation.
  - Prepare 1 mL aliquots and store at −20°C until use.

- 2 × Spotting buffer
  - Add:

    2 mL of 100 mM sodium borate buffer, pH 9.0
    20 μL of 5% sodium azide
    0.5 mL of 10% dextran stock solution
    10 μL of 1% Igepal stock solution.

  - Adjust volume to 10 mL with H_2O.
  - Filter for sterilisation.
  - Prepare aliquots and store at −20°C until use.
- Blocking buffer
  - Add:

    40 g skimmed milk powder
    100 mL of 10× PBS
    2 mL 5% sodium azide stock solution
    5 mL 20% Tween-20 stock solution

  - Adjust volume to 1 L with H_2O.
  - Mix well for at least 30 min on a magnetic stirrer in order to allow the milk powder to dissolve completely.
  - Store at 4°C and use within a few days.
- Washing buffer A
  - Add:

    100 mL of 10× PBS
    2 mL 5% sodium azide stock solution
    2.5 mL 20% Tween-20 stock solution
    2.5 mL 20% Triton-X100 stock solution

  - Adjust to 1 L with H_2O.
  - Mix for at least 30 min on a magnetic stirrer before usage.
- Washing buffer B
  - Add 5 mL 10× PBS to 495 mL H_2O.
- Washing buffer C
  - Mix 100 mL methanol and 70 mL acetic acid. Adjust the volume to 1 L with H_2O.

33.3 Protocols

According to the experimental process, the protocols below are divided into four sections: antibody microarray production (Sect. 33.3.1), sample preparation and labelling (Sect. 33.3.2), incubation of the samples on the microarrays (Sect. 33.3.3) and finally image acquisition and data analysis (Sect. 33.3.4).

33.3.1 Antibody Microarray Production

33.3.1.1 Pre-processing and Storage of Antibodies

Perform all subsequent steps at 4°C or on ice.

- Purify antibodies delivered in an unpurified formulation such as ascites fluid, whole antiserum or in presence of stabilisers such as BSA or gelatine. Use Protein A or G columns (e.g. Pierce Nab Protein G Spin Kit, Thermo Fisher Scientific, Waltham, USA) depending on the exact host used for antibody production and antibody isotype. Follow the instructions of the respective user manual.
- For antibodies formulated in another buffer system or with the addition of glycerol, exchange buffer to PBS by dialysis (Pierce Slide-A-Lyzer, Thermo Fisher Scientific, Waltham, USA).
- If necessary, adjust antibody concentration to 2 mg/mL (see Note 1) by filtration (Microcon YM-100, Millipore, Schwalbach, Germany) or dialysis (Pierce Slide-A-Lyzer and Pierce Slide-A-Lyzer concentrating solution; Thermo Fisher Scientific, Waltham, USA).
- Prepare 5 μL aliquots (see Note 3) of antibody solution to avoid additional freeze–thaw cycles.
- Store antibody aliquots at −20°C until use.

33.3.1.2 Preparation of Antibody Spotting Microtiter Plates

Prepare the spotting microtitre plate(s) directly prior to microarray spotting. Handle all tubes and plates on ice.

1. Thaw antibodies on ice.
2. Mix 5 μL antibody with 5 μL 2× spotting buffer (see Note 3) in order to have a final spotting concentration of 1 mg/mL.
3. Transfer the mix to the appropriate wells of the spotting microtitre plate. Pipette carefully in order to prevent any air bubbles.
4. Include positional controls: add 0.25 mg fluorescently labelled protein, 0.75 mg BSA and 1× spotting buffer in a volume of 10 μL to wells of the spotting microtitre plate(s). Positional controls (Fig. 33.3a) facilitate easy identification

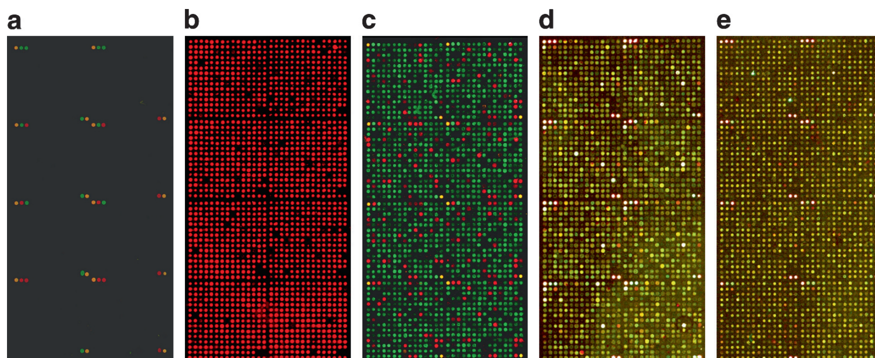

Fig. 33.3 Quality control of an antibody microarray consisting of 1,800 features. Antibodies have been spotted in duplicates. (**a**) Two-colour positional controls facilitate easy tracking of different grids and verification of spot segmentation. (**b**) Sypro Ruby staining acts as a quality control measure for spotting. Negative controls do not show any immobilised protein. Panel (**c**) shows an antibody microarray incubated with 5 nM each of secondary fluorescently labelled antibodies against rabbit IgG (*green*) and mouse IgG (*red*). The majority of antibodies on the microarray were produced in rabbit. Panel (**d**) presents an antibody microarray that was incubated with two human plasma samples following the protocols presented here. In (**e**), an antibody microarray was incubated with proteins extracted from two related human urine samples according to the protocols presented here

of slide orientation and grid recognition as well as spot segmentation during image processing.

5. Include negative controls: add 5 µL 2× spotting buffer and 5 µL PBS to some wells of the spotting microtitre plate(s).
6. Mix liquid in the microtitre plate wells thoroughly using a plate vortexer.
7. Centrifuge plate(s) at 1,000 g for 2 min and keep at 4°C covered with a lid until array spotting.

33.3.1.3 Test Spotting for Parameter Optimisation (optional)

Optionally a test-spotting run can be performed in order to assess the performance of the existing infrastructure. The test system should mimic the final production run with regard to number of antibodies, complexity of the arrays as well as duration of spotting.

1. Prepare spotting plates according to Sect. 33.3.1.2. Instead of antibodies, however, use BSA in the same concentration (see Note 4). Integrate three consecutive negative controls every 20 spots.
2. Spot the test plates according to protocol 33.3.1.4 with all spotting settings and type and number of slides being identical to the real spotting run.
3. Stain part of the produced slides picked from different positions within the spotter according to protocol 33.3.1.6.

4. Make sure that spots are clearly separated on the arrays as well as the pre-spotting slides.
5. Make sure that protein has been immobilised at all positions to which BSA was spotted (see Note 5).
6. Make sure that there is no carryover of protein to the negative controls (see Note 6).
7. Make sure that each spot shows a homogenous spot morphology (see Note 7).
8. Make sure that spot morphology and intensity are consistent throughout the array (see Note 8).

33.3.1.4 Spotting Process

1. Program the robot and fill the washing buffer reservoirs and the air humidifier. If the robot has a cooling system, set it to a temperature of 10°C.
2. For pin spotters, clean the pin tool and the pins thoroughly (Note 9). Follow the manual of the pin manufacturer.
3. Start a pre-spotting by delivering at least 1,000 spots of 1 × spotting buffer containing 1.0 mg/mL BSA. Make sure that all pins or piezo needles are performing well.
4. Place the slides in the robot using powder free nitrile gloves. Pay attention not to touch the slides on the surface.
5. Place the spotting microtitre plates in the robot. If the robot has no cooling device, allow the plates to adapt to room temperature beforehand.
6. Allow the relative humidity in the robot to reach a value of about 50%.
7. Start the spotting process.
8. After spotting is finished, keep slides for two more hours within the robot at 50% humidity.
9. Label slides with a diamond marker and place them in a slide rack.
10. Leave the slides overnight at 4°C in the dark.

33.3.1.5 Post Processing

1. Allow slides to adapt to room temperature for 30 min.
2. Perform quality control analysis (Protocol 33.3.1.6) for a part of the slides picked at random from different positions within the microarraying robot.
3. Wash slides three times in washing buffer A by quickly moving the rack up and down.
4. Incubate slides in a large volume of blocking buffer for at least 4 h at 4°C with gentle agitation.
5. Wash slides 4 × 5 min in washing buffer A.
6. Wash slides 2 × 5 min in washing buffer B.
7. Dry each slide individually by aiming a sharp stream of air to each spotting area. Always keep slides wet beforehand and do not allow remaining droplets to move into the spotting area.
8. Store slides in a humidity chamber at 4°C for up to 12 months.

33.3.1.6 Quality Control

For quality control, immobilised proteins can be stained after microarray production by a fluorescent dye such as Sypro Ruby (Fig. 33.3b). It is essential to perform such staining prior to blocking the slide surface.

1. Wash slides 4 $\times$ 5 min on a shaker in washing buffer A.
2. Cover slides for 1 h with the ready-to-use Sypro Ruby staining solution.
3. Wash slides 4 $\times$ 5 min in washing buffer C.
4. Wash slides 2 $\times$ 5 min with H_2O.
5. Dry each slide individually by pointing a sharp stream of air to each spotting area. Always keep slides wet beforehand and do not allow remaining droplets to move into the spotting area.
6. Scan the slides with a microarray fluorescence scanner recording the emission at 610 nm using excitation at 280 nm or 450 nm.

As an additional control, antibody microarrays can be incubated with 10 nM fluorescently labelled secondary antibodies (Fig. 33.3c) in blocking buffer for 2 h to control protein immobilisation and functionality.

33.3.2 Sample Preparation

33.3.2.1 Sampling and General Remarks

Attention: Human samples should be handled as biohazards.
In general, all protein samples should be thawed and handled on ice in order to prevent degradation by proteases.

For antibody microarray experiments, plasma, serum or urine samples can be used as well as samples which are derived from extractions from cell culture or fresh frozen tissue. The exact protocol for protein extraction from cell culture or tissue will largely depend on the sample type as well as on the respective project. Therefore, only general advice can be given here:

- In general, a protein concentration of at least 5 mg/mL should be obtained and the final buffer must not contain ammonium ions or primary amines (e.g. Tris, glycine, urea, guadinine).
- Aliquot samples as soon as possible and avoid repeated freeze-thaw cycles.
- Sample handling has a major effect on the protein quality and composition. Therefore, treat all samples within an experiment series in a uniform manner. Make sure that this happens also prior to their arrival in the microarray laboratory, e.g. during sampling. Factors that could affect quality are the kind of columns used for plasma/serum preparation, the period that a sample remains at room temperature prior to freezing, time of adding protease inhibitors and the number of freeze-thaw cycles, for example.
- Measure protein concentrations prior to labelling by the bicinchoninic acid (BCA) assay in replicates, using BSA as a standard

33.3.2.2 Labelling of Protein Samples

This protocol is used for detection strategies based on hapten or fluorescent dye labelling. Labels are covalently attached to amino groups of the proteins using NHS-ester chemistry. For one-colour assays, all samples are labelled with the same reagent. For competitive two-colour assays, samples are labelled by two different reagents and incubated on the same array.

1. Thaw protein samples on ice; the protein concentration should be at least 5 mg/mL.
2. Transfer 1 mg of each protein sample to a 1.5 mL reaction tube (see Note 10).
3. Adjust volume to 200 μL with H_2O.
4. Per reaction, add 37.5 μL from a master mix consisting of
   - 12.5 μL 20% Triton stock solution
   - 25 μL 1 M sodium bicarbonate stock (pH 9.0).
5. Dissolve label reagent (NHS ester of hapten or fluorescent dye) in H_2O to have a concentration of 8 mM. Mix thoroughly by pipetting. Use label reagents immediately after dissolving them in order to prevent hydrolysis of the NHS-esters.
6. Per reaction, add 12.5 μL of dissolved label reagent.
7. Incubate reaction tubes for one hour on a shaker (200 rpm) at 4°C, protected from light.
8. To stop the reaction, add 17 μL of 50% hydroxylamine to each reaction tube and incubate for 30 min at 4°C.
9. Use Zeba 2 mL Desalt columns (Pierce) in order to remove unreacted dye and exchange buffer to PBS according to the protocol provided by the manufacturer.
10. Add 30 μL of 25 × protease inhibitor cocktail stock solution.
11. Prepare 30 μL aliquots and store protected from light at −20°C.

33.3.2.3 Measuring of Label Ratio

With the protocol listed below, the specific labelling can be measured easily for protein samples labelled with a fluorescent dye. Biotin labelling can be assessed by a biotin quantification kit (Thermo Fisher Scientific, Waltham, USA).

1. Add 30 μL of a labelled protein sample to 570 μL PBS.
2. Measure the absorbance at 280 nm.
3. Measure the absorbance at the maximal absorption of the dye (553 nm for Dy-549 and 655 nm for Dy-649).
4. Calculate the average degree of specific labelling of the protein sample according to equation 33.1. Use the molar extinction coefficient (ε_{Dye}) and correction factor (cf) provided for the respective dye (e.g. $\varepsilon_{Dy549} = 150{,}000\ M^{-1}\ cm^{-1}$, $\varepsilon_{Dy649} = 250{,}000\ M^{-1}\ cm^{-1}$, $cf_{Dy549} = 0.08$, $cf_{Dy649} = 0.05$). Use the value of

albumin as an averaged molar extinction coefficient for the protein sample ($\varepsilon_{Protein} = 43{,}824\ M^{-1}\ cm^{-1}$).

$$Ratio = c_{Dye} \div c_{Protein} = \frac{A_{Dye}}{\varepsilon_{Dye}} \div \frac{A_{280} - (cf \cdot A_{Dye})}{\varepsilon_{Protein}} = \frac{A_{Dye}}{A_{280} - (cf \cdot A_{Dye})} \cdot \frac{\varepsilon_{Protein}}{\varepsilon_{Dye}} \quad (33.1)$$

5. Repeat label reaction for samples having a specific labelling of less than 75% or more than 125% of the median of all samples within an experimental series (Note 11).

33.3.3 Sample Incubation

Typical results for dual-colour incubations are shown for human plasma (Fig. 33.3d) and human urine (Fig. 33.3e). The protocol below is adapted to samples labelled by a **fluorescent dye**:

1. Attach home-made plexiglas incubation chambers to the slide using a double-adhesive tape (see Note 2).
2. Place the slides on a slidebooster instrument and start mixing (see Note 12).
3. Pre-block arrays for 2 h using blocking buffer (see Note 13). Adjust the blocking volume to fill the chamber.
4. Prepare incubation buffer by adding to the blocking buffer Tween-20 to a final concentration of 1%.
5. Dilute labelled protein samples 1:10 in the incubation buffer. For a two-colour approach, dilute each of the two samples (e.g. mix 60 μL sample A, 60 μL sample B and 480 μL incubation buffer).
6. Exchange blocking buffer with the respective incubation mix. Adjust the incubation volume to fill the chamber to a height of 3–4 mm.
7. Cover incubation chambers with a coverslip and incubate for 14 h.
8. Take off incubation mix and wash each array 4 × 5 min with washing buffer A under stirring conditions. Add the washing buffer quickly in order to keep the slide surface wet.
9. Stop slidebooster and place slides in a container filled with washing buffer A.
10. Detach incubation chambers. Take care to remove all remaining residues of adhesives and keep the array surface wet during removal.
11. Immediately place arrays in a slide rack in a container filled with washing buffer A and wash for 3 × 5 min.
12. Wash 2 × 5 min in washing buffer B.
13. Dry each slide individually by aiming a sharp air stream at the spotting area. Always keep slides wet beforehand and do not allow remaining droplets to move into the spotting area.
14. Store slides protected from light until scanning.

For samples labelled with a **hapten,** interrupt protocol after step 7 and continue as follows:

- Wash the arrays 6 × 5 min with blocking buffer supplemented with 0.05% Tween-20 and 0.05% Triton-X100.
- Incubate with 10 nM fluorescently labelled anti-hapten antibody in blocking buffer for 1 h.
- Continue with step 8 of the protocol above.

For a **sandwich strategy**, adjust the protein concentration of the unlabelled sample in the incubation mix to 0.25 mg/mL (step 5). Interrupt the protocol above after step 7 and continue as follows:

- Wash the arrays 6 × 5 min with blocking buffer supplemented with 0.05% Tween-20 and 0.05% Triton-X100.
- Incubate with the second set of analyte-specific detection antibodies in blocking buffer. The concentration should be adjusted in advance for each respective antibody.
- Wash the arrays 6 × 5 min with blocking buffer supplemented with 0.05% Tween-20 and 0.05% Triton-X100.
- Incubate with the respective fluorescently labelled secondary antibodies for 1 h at a concentration of 10 nM in blocking buffer.
- Continue with step 8 of the protocol above.

33.3.4 Data Analysis

- Detect signal intensities in a microarray scanner. Adjust the scanner settings of the photomultiplier tube (PMT) and the laser power (LP) beforehand in order to obtain visible signals for most spots with only a small number of saturated spots for abundant proteins (see Note 14). For two-colour incubations, adjust scanner settings additionally in a way that the signal intensity distributions for both dyes match each other. Keep scanner settings fixed for all arrays within an experimental series.
- Convert recorded image files into signal intensities by a software for semi-automatic spot recognition as well as signal quantification such as GenePix Pro, Mapix or the freeware TIGR Spotfinder.
- Import data into appropriate software for data analysis (see Note 15).
- Use the signal mean and subtract the median of the local background.
- For two-colour assays, calculate the ratio of the two colour channels and log transform the data.
- For two-colour arrays, adjust the colour channels through Lowess normalisation. For one-colour arrays, normalise signal intensity distributions between different arrays.

- Use methods such as linear models, SAM, ANOVA, clustering methods, principal component analysis or correspondence analysis to identify proteins that are differentially expressed and affiliate difference to biological factors.

33.4 Notes

1. *Antibodies*: Antibodies, which are working with a high specificity and sensitivity in Western and ELISA can be used for antibody microarray experiments. Western blotting is the current method of choice to assess the specificity of antibodies. Antibodies should be purified and formulated in PBS without addition of stabilisers such as BSA, gelatine or glycerol, which are negatively affecting the spotting process. It is beneficial to immobilise the antibodies in a comparably high concentration of 1 mg/mL after addition of the spotting buffer (Kusnezow et al. 2003). However, it is possible to immobilise antibodies in lower concentrations, if sensitivity is of minor importance than antibody consumption.
2. *Incubation chamber*: Also LifterSlips or Gene Frames (Thermo Fisher Scientific, Waltham, USA) can be used to keep the incubation volume. However, higher sensitivity will be obtained with the increased volumes that were made possible by an adapted, homemade incubation chamber.
3. *Spotting volume*: The volume used in the spotting plates is depending on the spotting robot and the number of replicates and slides. For one spot, usually between 0.5 and 10 nL are used depending on pin size (contact printing) or number of drops (non contact printing). The volume in each sample uptake is 0.25–1.25 μL for pin spotters. Usually, the minimum volume which can be handled by most microarraying robots is around 5 μL. A volume of 15 μL should be highly sufficient for most spotting projects.
4. *Fluorescently labelled protein in test runs*: Fluorescently labelled proteins should not be used to optimise the spotting process. Their increased hydrophobicity due to the dye would hide problems that could occur with antibodies and would introduce artefacts, which would never occur with unlabelled proteins. Therefore, it is recommended to use unlabelled BSA for optimising the spotting parameters in a test run and to visualise immobilised proteins by subsequent staining with Sypro Ruby (Sect. 33.3.1.6).
5. *Spots are missing*: If random spots are missing, in most cases, spotting pins have got stuck in the pin tool. Clean and dry pins and pin tool thoroughly. If the problem is persistent, decrease the humidity in the spotter to a value of 35–45%. If spotting stops after a certain number of replicates or samples perform an intensive pin cleaning protocol using special reagents according to the protocol of the pin manufacturer (e.g. http://arrayit.com/Products/MicroarrayI/PPCK80/ppck80.html). If problems persist, increase pin washing time between sample uptakes, reload pins more often or increase concentration

of detergent in the spotting buffer. If spots are observed optically after array production by making them visible by "breathing" upon the surface, but no or few proteins are detected after staining, make sure that the epoxy groups of the array surface are still active. Avoid Tris or betaine addition in the spotting solution.

6. *Carryover*: If carryover is observed, increase washing time between sample uptakes and make sure that pins are completely dried after washing. Exchange washing buffer more often, add detergents (e.g. 0.1% Tween-20) or use additional sonication.
7. *Inhomogeneous spot morphology*: To avoid doughnut-shaped spots, increase humidity during spotting process or add compounds to the spotting solution, which reduce the evaporation speed or increase surface tension. To avoid blurry spots, increase detergent concentration of the spotting buffer.
8. *Inconsistent spot morphology*: Make sure that formulation and concentration of antibodies is consistent. If there is a systematic pattern, test the surface coating prior to spotting by breathing carefully upon the surface. The vapour should be completely homogenous. Additionally, surface inhomogeneities (which sometimes pass quality control by the manufacturers) can be detected by scanning a slide at full laser power and PMT.
9. *Pin handling*: Always use powder-free nitrile gloves for handling pins and pin-tool. Clean pin tool and pins exactly according to the manual of the manufacturer. In the last step dip them in 100 % ethanol and completely dry them using a stream of air. Do not use pressurised air canisters, which might contain organic propellants.
10. *Downscaling of labelling reaction*: It is possible to perform label reactions with less starting material of your sample, although reproducibility may suffer. Reduce the overall reaction volume but keep the concentrations of protein and label reagent. Eventually, use smaller columns for the removal of unreacted dye.
11. *Label ratio*: If the degrees of specific labelling of the samples differ substantially, check again the protein concentration of the starting materials. Also, note that the amount of dye can differ from the value given for different aliquots by the manufacturer. In addition, the reactivity of dyes may differ between batches. Therefore, it is recommended to label large sets of samples simultaneously with the same solution of label reagent.
12. *Slidebooster*: If no slidebooster instrument is available, incubations can be performed under non-mixing conditions. However, incubation times should be extended and lower sensitivities will be obtained (Kusnezow et al. 2006a, b).
13. *Blocking and incubation buffer*: If increased background is observed, use "The blocking solution" (Candor Biosciences GmbH, Weißensberg, Germany) for step 3 and after addition of Tween-20 in step 4 of the sample incubation protocol.
14. *Combining information derived from two scanner settings*: If it is not possible to obtain a representative majority of spots from one scanner setting, it is possible to perform multiple scans at different intensities and combine them using Masliner prior to data analysis (Dudley et al. 2002).

15. *Software for data analysis*: Many very versatile tools for the normalisation, filtering and statistical testing are available within the Bioconductor package (Gentleman et al. 2004) for R. Most important functions are also integrated in the online analysis platform Expression Profiler (Kapushesky et al. 2004). Also, the freeware TIGR MultiExperiment Viewer (Saeed et al. 2003) allows application of many different analysis algorithms to the data. We used M-CHiPS (Fellenberg et al. 2006). M-CHiPS is well-suited especially for correspondence analysis and for correlating the samples to all given biological factors in one go. In addition there is a variety of commercial tools available for data analysis.

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