Chapter 7

High-Throughput Immunofluorescence Microscopy Using Yeast Spheroplast Cell-Based Microarrays

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Abstract

We have described a protocol for performing high-throughput immunofluorescence microscopy on microarrays of yeast cells. This approach employs immunostaining of spheroplasted yeast cells printed as high-density cell microarrays, followed by imaging using automated microscopy. A yeast spheroplast microarray can contain more than 5,000 printed spots, each containing cells from a given yeast strain, and is thus suitable for genome-wide screens focusing on single cell phenotypes, such as systematic localization or co-localization studies or genetic assays for genes affecting probed targets. We demonstrate the use of yeast spheroplast microarrays to probe microtubule and spindle defects across a collection of yeast strains harboring tetracycline-down-regulatable alleles of essential genes.

Key words: Yeast, immunofluorescence, high-throughput microscopy, cell microarrays, microtubule.

1. Introduction

DNA microarrays and mass spectrometry, among other approaches, have proved to be powerful tools for direct assay of gene function on a genome-wide scale (1, 2). However, such approaches often do not monitor gene and protein behavior in intact cells nor do they assay behavior at the single cell level (3). Nonetheless, it is often desirable to measure single cell phenotypes, and high-throughput microscopy offers a viable strategy for such assays, especially when used in combination with available libraries of genetically distinct cells. To aid in performing such assays, we have described an extension of the spotted cell

E. Palmer (ed.), *Cell-Based Microarrays*, Methods in Molecular Biology 706,
DOI 10.1007/978-1-61737-970-3_7,

microarray platform (4) to perform high-throughput immunofluorescence assays on yeast cells. This approach, dubbed yeast spheroplast microarrays, enables analysis of localization phenotypes of probes of interest (e.g., any target of an affinity reagent such as an antibody) in thousands of genetically distinct cells in parallel.

Yeast spheroplast microarrays have several advantages compared with high-throughput assays in 96- or 384-well plates. First, the high capacity of the arrays (up to ~5,000 spots per array) minimizes the use of expensive reagents to screen a library of cells by limiting the use of antibodies or dyes to single microscope slides. Second, the high density of samples in the arrays increases data acquisition speed by automated microscopy. Third, yeast spheroplast microarrays provide a platform for highly parallel and reproducible large-scale screens. More than a hundred cell microarrays can be manufactured from the same sample sources under the same conditions, and microarrays can be stored at –80°C at least for a month without apparent loss of immunostaining signals. As each slide can in principle be probed with a different antibody, this allows many independent imaging assays to be performed on cells drawn from the same biological samples.

Performing such high-throughput immunofluorescence experiments in yeast requires spheroplasting the cells to allow antibody access to intracellular contents. One of the primary limitations of high-throughput immunofluorescence imaging of yeast cells is the difficulty associated with uniformly spheroplasting cells in a strain collection using a single spheroplasting condition, especially given that optimal treatment times can vary with genetic background. Therefore, spheroplast conditions must be optimized by testing the time of zymolyase treatment when manufacturing arrays from different yeast cell libraries.

To enable such experiments, we present here a protocol for high-throughput spheroplasting of yeast cells and construction and imaging of spheroplast cell microarrays (*see* **Fig.** 7.1 for an overview of the protocol). As a demonstration of the construction and utility of such arrays, yeast spheroplast chips were built from the *TetO7* promoter collection covering 75% of essential genes (5), then probed with anti-alpha tubulin antibody to examine the effects on the organization of microtubule and spindle when essential genes were downregulated (**Fig.** 7.2).

Yeast microtubule organization depends strongly on cell cycle progression (6, 7). In wild-type G1-phase cells, microtubules form star-like cytoplasmic arrays radiating from the microtubule organizing center (**Fig.** 7.3a). During S/G2 phase, cytoplasmic astral microtubules reorient towards the bud tip and penetrate the bud (**Fig.** 7.3b). When cells enter mitosis, microtubules rearrange into a bipolar spindle positioned through the bud neck. In anaphase, cytoplasmic microtubules shorten, the spindle

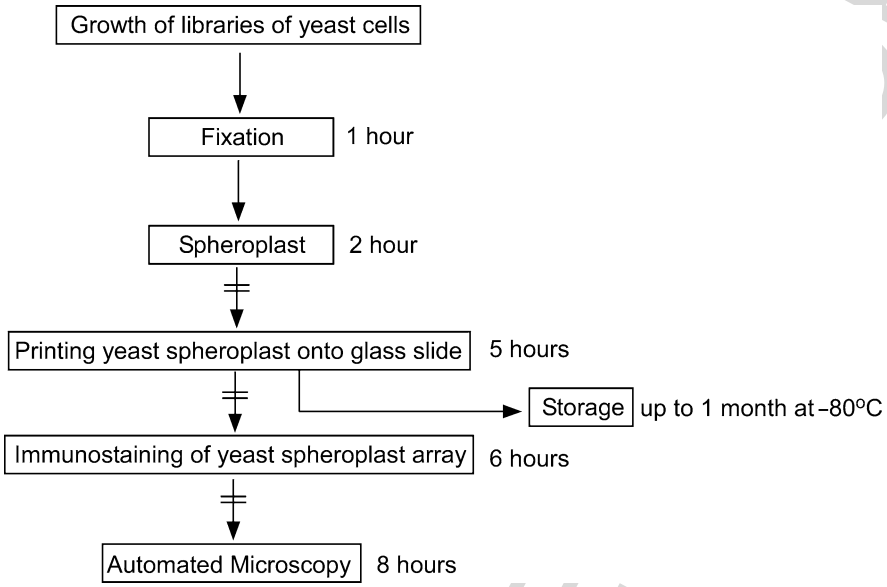

Fig. 7.1. Work flow of the yeast spheroplast microarray protocol. Yeast cells are cultured, fixed, and spheroplasted in 96-well plates, e.g., using an automated liquid handling robot. Subsequently, they are robotically printed onto poly-L-lysine microscope slides using a slotted steel pin-based DNA microarray robot. The resulting slides each contain ~5,000 spots. Each slide can be stored at –80°C or can be probed with a specific antibody immediately after printing. Images are acquired through automated microscopy. The different steps of the protocol are indicated in *boxes* by *arrows*. The approximate time required for each step is indicated near the *box*. Steps at which the protocol can be paused are indicated in the diagram by pairs of *short lines* across the *arrows*. Times indicated for each step are approximate and will depend upon the precise equipment used for the experiment.

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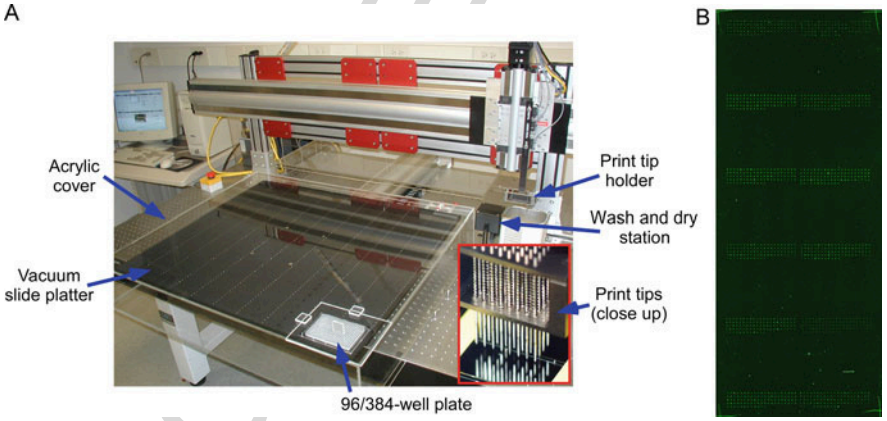

Fig. 7.2. A microarray printing robot suitable for printing yeast spheroplast microarrays, shown alongside an example microarray (**a**). A Stanford-style Newport DNA microarray print robot with 12 pins directly delivers yeast spheroplasts from 96-well plate onto slides (**a**) (4). A yeast spheroplast array carrying ~700 yeast *Tet07*-promoter strains (spot diameter, 200 μm; spot-to-spot distance, 410 μm) is shown in (**b**).

elongates, and sister chromosomes are segregated (**Fig.** 7.3c). When cytokinesis is completed, the spindle disassembles (**Fig.** 7.3d).

In the example spheroplast array, abnormal microtubule structures could be observed in cells with knockdown of genes

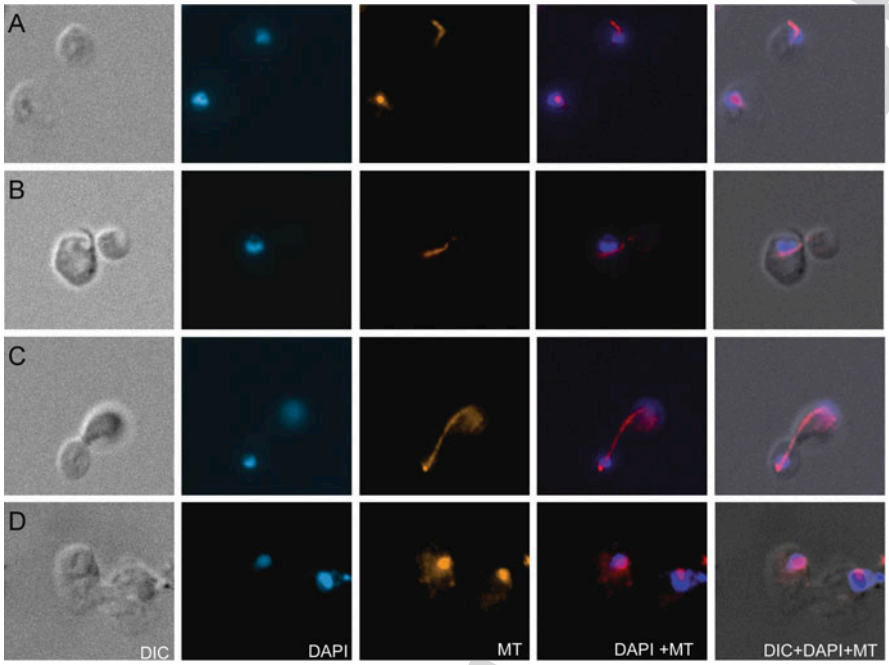

Fig. 7.3. Examples of microtubule and spindle morphology in different yeast cell cycle stages from (**a**) to (**d**) show G1 phase cells, S/G2 phase cells, anaphase cells, and completed cytokinesis, respectively. Nuclei and microtubules are depicted.

whose mutations are known to cause defective spindle formation. For example, Spc97 and Spc98 are two major components of the microtubule nucleating Tub4 complex, involved in microtubule nucleation and mitotic spindle organization (8). Downregulation of *SPC97* and *SPC98* resulted in short spindles, elongated cytoplasmic microtubules, and defective

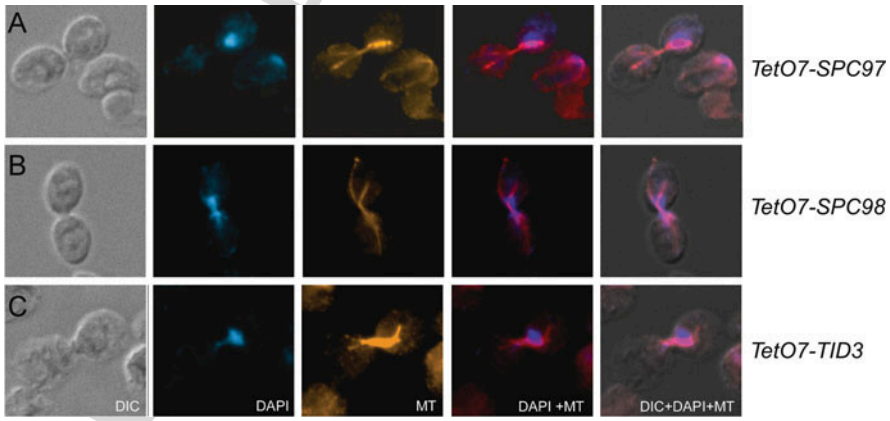

Fig. 7.4. Example images for *TetO7* promoter strains exhibiting defective microtubule and spindle structure (**a**, **b**). Downregulation of *SPC97* and *SPC98* resulted in short spindles and elongated cytoplasmic microtubules (**c**). Downregulation of *TID3* caused defective spindle elongation and uneven nuclear division.

chromosome segregation, thus showing a defect resembling that of the corresponding temperature-sensitive mutants (**Fig.** 7.4a,b). Similarly, Tid3 is a component of the kinetochore-associated Ndc80 complex, involved in chromosome segregation and microtubule nucleation (9, 10). Downregulation of *TID3* caused defects in spindle elongation and nuclear division (**Fig.** 7.4c). Thus, the potential can be seen of spheroplast chips for high-throughput immunofluorescence imaging-based genetic screens in yeast.

2. Materials

2.1. Yeast Cell Growth and Fixation

1. Yeast strains: for the example array shown here, we used the yeast TetO7-promoter strain collection (5) (Open Biosystems).
2. Growth medium: YPED medium (1% yeast extract, 2% peptone, and 2% D-dextrose) supplemented with 200 μg/ml doxycycline.
3. 37% Formaldehyde.
4. 1× PBS buffer: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 ml of distilled H_2O. Adjust pH to 7.4 with HCl. Add H_2O to 1 l.
5. Spheroplast buffer: 1.2 M sorbitol, 0.1 M KH_2PO_4, pH 7.5.

2.2. Zymolase Treatment

1. Zymolase solution: Spheroplast buffer supplemented with 0.025 mg/ml zymolyase 20T (Seikagaku corporation) and 0.4 μl β-mercaptoethanol.

2.3. Preparation of Poly-L-Lysine Glass Slides

1. Glass slide wash solution**:** dissolve 33 g NaOH pellets in 133 ml H_2O, add additional NaOH pellets until solution is saturated, and then add 200 ml 95% EtOH.
2. Poly-L-lysine solution**:** 250 ml H_2O, 35 ml 1× PBS (filtered), 50 ml poly-L-lysine.
3. Glass slide dish.
4. Glass slide rack.

2.4. Spheroplast Microarray Printing

1. DNA microarray printing robot. Here, we use a Stanford-style Newport printer.
2. Printer pins. Here, we use conically tapered 1/16 inch diameter stainless steel printing tips with 0.0015 inch slots (Majer Precision Engineering).
3. 0.5× SSC.

2.5. Storage and Recovery of Yeast Spheroplast Microarrays

1. 95% Ethanol.

2.6. Immunostaining of Yeast Spheroplast Microarrays

1. Methanol, –20°C.
2. Acetone, –20°C.
3. Blocking buffer: 3% BSA in 1× PBS.
4. Primary antibody: Anti-bovine α-tubulin, mouse monoclonal antibody, and 4 μg/ml in blocking buffer.
5. Secondary antibody: Texas-red conjugated goat anti mouse IgG (H+L), 4 μg/ml in blocking buffer.
6. VECTASHIELD hard set mounting medium (Vector Laboratories, Inc) with DAPI.
7. Clear nail polish.
8. Staining jars.

2.7. Automated Imaging

1. Automated fluorescence microscope.

3. Methods

3.1. Yeast Cell Growth and Fixation

1. Grow or treat cells in 96-well plates in 170 μl proper medium.
2. Fix cells immediately after growth or treatment in proper growth medium with 1/10 volume 37% formaldehyde for 1 h at 30°C.
3. Wash cells with 1× PBS buffer twice and resuspend cells in 200 μl spheroplast buffer.
4. At this point, cells can be stored at 4°C overnight or spheroplasted immediately as in **Section 3.2**.

3.2. Zymolyase Treatment

1. Spin down cells and resuspend cells in 200 μl zymolase solution (*see* **Note 1**).
2. Incubate cells for 2 h at 30°C. This is a critical step and should be monitored closely. *See* **Fig.** 7.5 for images of the desired degree of spheroplasting.
3. Centrifuge cells at low speeds (<3,000 rpm) and wash cells twice with spheroplast buffer, and resuspend cells in 200 μl spheroplast buffer.

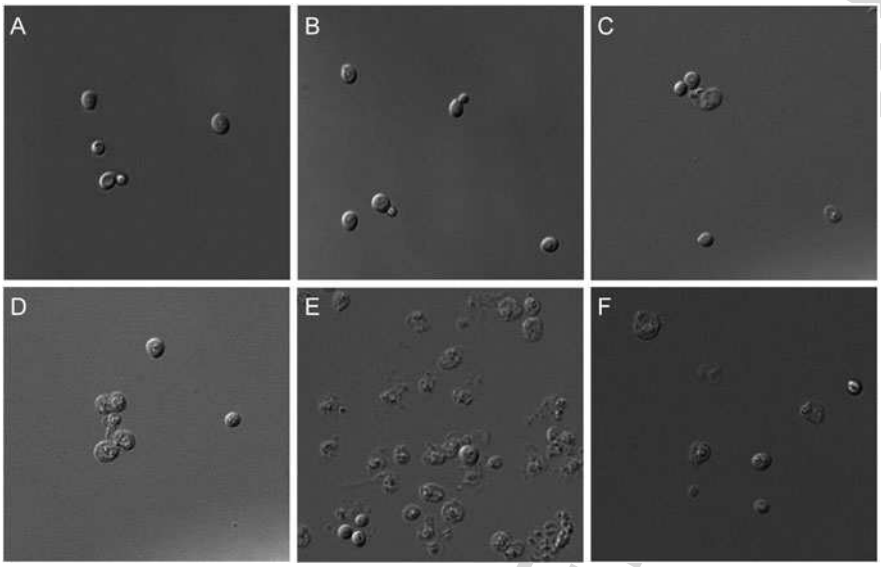

Fig. 7.5. Examples of morphology changes of yeast cells treated with zymolyase to differing extents. Cells should be a *dark*, translucent *gray* after an appropriate degree of digestion (**d**). (11). *Bright cells* are insufficiently digested (**a**–**c**) (11). "Ghost cells" (*pale gray* with little any internal structure) have been over-digested (**e**, **f**) (11). Spheroplasting protocols for cells typically employ a relatively high concentration of zymolyase and short digestion times (less than 30 min). However, given that yeast spheroplast microarrays are designed for large-scale experiments usually analyzing hundreds or thousands strains at a time, 30 min digestion times are impractical and unforgiving for small errors in timing. We therefore employed lower concentrations of zymolyase and longer digestion times in order to get an appropriate degree of digestion. The optimal concentration for the Tet07 strain collection in our example assay was 0.025 mg/ml; the digestion time was 2 h (**d**).

4. At this point, spheroplasted cells can be stored at 4°C overnight or printed immediately as in **Section 3.3** (*see* **Note 2**).

3.3. Preparation of Poly-L -Lysine Glass Slides (Described for 30 Slides)

1. Rinse slide dishes completely with H_2O in a plastic basin four times to wash away any dust and debris that might be on the slides (*see* **Note 3**).
2. Place racks in glass slide dishes and pour slide wash solution over slides and cover. Shake gently for 2 h (*see* **Note 4**).
3. Rinse each rack with tap-distilled water two times, making sure to rinse both surfaces of each slide.
4. Rinse the racks with double distilled H_2O at least two times. Make sure that you have removed all of the NaOH.
5. Place one rack of slides into glass slide dishes, draining briefly, and pour poly-L-lysine solution to cover each set of slides. Shake gently for 30 min.
6. Rinse each rack with distilled H_2O twice in a plastic basin by removing poly-L-lysine from slides.

7. Place racks on top of 2 folded over paper towels in a tabletop, centrifuge, and spin at 600 rpm at room temperature for 5 min to dry.
8. Store slides in a clean plastic box at room temperature.

3.4. Spheroplast Microarray Printing

In principle, any slotted pin-based DNA microarray printer can be used. We present specific protocols for the Stanford-style Newport printer, using Arraymaker control software.

1. Depending on the array printer, it may be necessary to set the print depth to be appropriate for 96-well plates prior to loading a plate in the plate holder (*see* **Note 5**).
2. Use 12 pins, skipping a pin in each direction. Pins are placed in hole numbers 2, 4, 10, 12, 18, 20, 26, 28, 34, 36, 42, and 44 (*see* **Note 6**).
3. Position poly-L-lysine-coated slides on the printing platter, taping in position if necessary.
4. Fill sonicating water bath with 0.5×SSC.
5. For plate alignment, due to the variability in well volumes, align printer pins so that the liquid level is just below the start of the beveled portion of the printing tip.
6. Align the slide position as typical for DNA microarray printing.
7. Set wash time at 6,000 ms, dry time at 8,000 ms, load time at 1,000 ms, the spot-to-spot distance as 410 μm. Use "slow pickup."
8. Test print by using a test plate with the wild-type samples before the large-scale printing, and check the cell counts under a microscope (*see* **Note 7**).
9. Start the formal print runs once the test print results are satisfying.
10. When the print is complete, tape slides into a swinging bucket plate centrifuge (max: 5 slides/bucket) or use custom-made plastic flat slide holders, and spin flat at 1,500–2,000 rpm for 5 min.

3.5. Storage and Recovery of Yeast Spheroplast Microarrays

1. Store spheroplast chips at 4°C for overnight or at –80°C for long-term storage (**Fig.** 7.6) (*see* **Note 8**).
2. To recover a slide after storage at –80°C, fill a 50 ml Falcon tube with 95% EtOH. Dunk slide into the tube immediately upon taking slide out of the freezer (*see* **Note 9**).
3. Take the slide out quickly and put it into a clean and dry 50-ml Falcon tube, and then dry the slide by spinning in an empty 50-ml tube without cap at 600–700 rpm for 5 min.

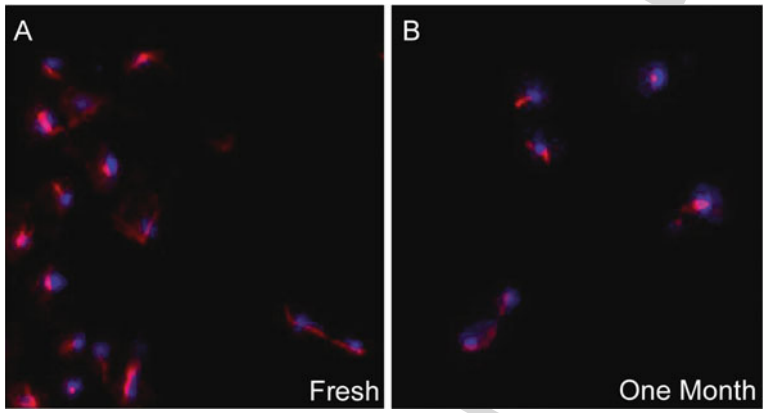

Fig. 7.6. Spheroplast microarrays can be stored at least a month at –80°C without substantial effects on cellular morphology or subcellular structure, as shown here for microtubule structure. Compared to fresh chips (**a**), chips stored at –80°C still maintain intact microtubule structures after one month (**b**). Nuclei and microtubules are depicted.

3.6. Immunostaining of Yeast Spheroplast Microarrays

1. Permeabilize cells in cold methanol for 6 min and then in cold acetone for 30 s. Air-dry slides completely.
2. Wash slides for 5 min with 1× PBS.
3. Place ~200 µl blocking buffer on the slide. Trim a piece of parafilm to the size of the slide and gently lay over the sample. Incubate for 30 min at 30°C in a sealed humidity chamber (*see* **Note 10**).
4. Carefully remove the parafilm and add ~200 µl primary antibody onto the slide. For the anti-bovine α-tubulin mouse monoclonal antibody used as an example (Invitrogen, cat. no. A-11126). Incubate for 2 h at 30°C.
5. Wash the slide three times in staining jars with 1× PBS, 5 min each time.
6. Add ~200 µl secondary antibody onto the slides. Incubate for 2 h at 30°C and in the dark (*see* **Note 11**).
7. Wash the slide three times in staining jars with 1× PBS, 5 min each time.
8. Add ~60 µl VECTASHIELD hard-set mounting medium and cover with cover slip.
9. Let the mounting medium dry out for 15 min at room temperature or overnight at 4°C. Seal the slides with clear nail polish.
10. Store the slides at 4°C and protect the slides from light. Immunostained yeast spheroplast microarrays can be stored at 4°C for at least a month with no apparent loss of signal.

3.7. Automated Imaging

In principle, any automated fluorescent microscope can be used to capture cell chip images. Here, we have presented a protocol specific to the Nikon TE2000 inverted epifluorescence microscope with NIS Elements controller software.

1. Turn on the microscope and all components. Initialize the stage. With a dummy slide, calibrate the stage and slide mounting bracket to ensure a flat focal plane across the sample area on the slide. This can be accomplished by marking one side of the slide with reference marks in the corners of the slide (using, for example, a fine-point Sharpie marker), navigating to each mark, and adjusting the calibration screws in the mounting plate until all reference spots can be viewed clearly at the desired magnification by translating X and Y without adjusting focus.
2. Edit the create_block_coordinates macro script (*see* **Note 12**) to reflect the block size on the printed microarray. Block size is the number of rows and columns of spots printed by a single pin on the array. Also specify the spot pitch—the distance between the printed spots in the array construction (in this protocol, 410 μm was used in lines 24 and 25 of the script).
3. Mount the slide and manually scan to the first printed spot in the first block. Focus on the spot in DIC at the desired magnification. 60× or greater magnification is recommended.
4. Execute the create_block_coordinates macro. An ND Experiment file will be created that specifies the coordinates of all spots in the current block.
5. In the ND Experiment dialog box, specify the desired immunofluorescence wavelengths to be captured. Also specify whether to autofocus on every spot. This should be unnecessary if the stage and slide mounting bracket are well calibrated (*see* **Section 3.7**, Step 1). Finally, specify the output file.
6. Execute the ND Experiment. The microscope will image the current block.
7. Repeat Steps 3–6 for each block. A 12-pin print will have 12 blocks.

4. Notes

1. The spheroplast buffer with zymolyase and β-mercaptoethanol should be prepared fresh before use.

2. Note that yeast cells without cell walls are quite fragile, and in general spheroplasted cells should be handled carefully and centrifuged at low speeds.
3. We recommend wearing powder-free gloves at all times, as talcum powder will stick to slides and leave visible debris in the resulting images.
4. This wash removes residual oil and debris from slides. The wash solution should be prepared fresh before use.
5. On Stanford-style printers optimized for DNA microarray printing, this step is important, because 96-well plates are often taller than 384-well plates. If the vertical printing depth (Z) is not set high enough, the printing pins will hit the 96-well plate bottom.
6. If using the Stanford-style arrayer and Arraymaker software, be sure to use the 48-tip printing designation in the Arraymaker software.
7. Ideally, there should be ~20–30 cells per spot. If cell count is low, consider resuspending cells in smaller volumes.
8. Empirically, we find that spheroplast arrays are fine if stored at 4°C for a few days, but for longer periods, it is better to store them at –80°C. Storage times up to 1 month have been tested.
9. This step is to prevent condensation on the slide. Condensation will smear the spots, cause potential cross-contamination, and make it very difficult to grade the spots on the slide.
10. It is important to not let the slides dry out from this step on.
11. Keep slides in the dark from this point on to minimize photobleaching of fluors.
12. The create_block_coordinates script referenced in protocol **Section 3.7**, Step 2-on, suitable for use with NIS Elements microscope controller software (specifically tested on NIS Elements AR (Advanced Research) v3.0.)

```
//Automatic Spot Coordinate calculation for a spotted
 cell microarray
//Program moves in NORMAL PRINT PATTERN
//User inputs x,y,z at first, last spot
//Program calculates x,y,z of every spot
//by Traver Hart, 1/22/07

// BE SURE STAGE IS INITIALIZED BEFORE RUNNING THIS
   MACRO

char Debug[1024];
```

```
int main()
{

    double dYSpotBegin,dXSpotBegin, dZSpotBegin;
    double dYSpotEnd,dXSpotEnd, dZSpotTwo, dZSpotThree;
    double dYOffset,dXOffset,dyTemp,dxTemp, dZOffset,
     dZYOffset, dZXOffset, dzTemp;
    int i,j;
    int numRows, sectorWidth;

    sectorWidth = 21; // (y direction)
    numRows = 21;     // (x direction)

    //Set distance between spots - specified as 410
      microns in arrayer
    dXOffset=410;
    dYOffset=410;

    Int_Question("Automated Print Block ND
     Initialization","Please move to the TOP
    LEFT spot (should be position 1,1 of block).","OK"
      ,"","","",1,1);
    StgGetPos(&dXSpotBegin,&dYSpotBegin,&dZSpotBegin);

  dzTemp = dZSpotBegin;

     for(i=0;i<numRows;++i)
        {
          for(j=0;j<sectorWidth;++j)
          {
              dxTemp=(dXSpotBegin - (dXOffset*i) );
              dyTemp=(dYSpotBegin - (dYOffset*j) );

             ND_AppendMultipointPoint(dxTemp,dyTemp,
               dzTemp,"sample");
          }

        }

         _ND_CreateAndRunExperiment();
}
```

Acknowledgments

This work was supported by grants from the N.S.F., N.I.H., and Welch (F-1515) and Packard Foundations to E.M.M.

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Chapter 7

Q. No.	Query
AQ1	Please provide e-mail id for the contributors “Wei Niu and G. Traver Hart”.
AQ2	Please check sentence “Place racks on top. . . .” for clarity.
AQ3	Please check sentence “For the anti-bovine α-tubulin. . . . . .” for clarity.
AQ4	“Ster 3.7.1” has been changed to “Section 3.7, Step 1,”. Please check.
AQ5	“Ster 3.7.2” has been changed to “Section 3.7, Step 2.” Please check.