Genetics by microscopy

RNAi can unite the precision view from microscopy and the unbiased search from a genetic screen, said Ron Vale (University of California, San Francisco [UCSF], CA). The necessary tools for fly researchers are genomewide RNAi, high throughput microscopy, and some fancy image processing. “It’s a hard project for a single person, and it does take technology and a certain amount of money,” said Vale. “At the same time, whole genome screens are possible to do within individual labs. They do not need to be done within giant consortia.”

Vale has previously used RNAi to knock down the activity of 26 microtubule motors in the fly S2 cell line. At the meeting, he presented the results from a genomewide RNAi screen in S2 cells for proteins involved in spindle assembly. The numbers in the new screen were a lot higher: his group analyzed 14,400 genes and 4 million spindle images.

To reduce subjectivity and the required labor, the UCSF team used automated microscopy. Automated does not mean no work, however. “It takes a lot of tweaking to get right,” said Vale. “A lot didn’t work right out of the box.”

Setting up the screen involved preparing RNAs, testing the screen itself, and writing image-processing programs. The double-stranded RNAs came from a new library, which was designed by the UCSF team to minimize overlap with genes other than the intended target. Assay optimization was comprehensive—“more like something industry does to really troubleshoot all of the assay design so that it works on a large scale,” said Vale. A custom MATLAB program written by collaborators Roy Wollman and Jon Scholey (University of California, Davis, CA) identified spindle images, sent them to a special database designed by the team, and pulled out key numbers on spindle dimensions and composition.

A human had to come in at the end, however. For each gene, a researcher looked at the numbers and a panel of ~200 spindle images. “Seeing all the spindles arrayed side by side made it much easier,” said Vale.

Vale sees the screen as a success, especially given that “the spindle is a very well-trodden area.”

Attrition of the initial hits was heavy: less than 5% of weak hits and approximately 50% of strong hits were reproducible with an RNAi made to another part of the same gene. Furthermore, “this screen, like a genetic screen, is just the beginning,” said Vale. Secondary screens were used to understand and prioritize the remaining hits.

Those doing subsequent whole genome screens will have an easier time. The library created in this study is available from Open Biosystems both in the form of DNA template and dsRNA.

But the software will not be as easily translated to a new task, especially for those used to the uniformity of other types of analyses such as microarray experiments. “With image-based RNAi screening there may be some overlaps, but the algorithms for mitotic spindles are not directly exportable to another screen for lysosomes or cell shape,” said Vale. “You learn tricks for one that are useful for the next, but it’s still a big project.” Once the programming is done, however, a genomic screen can be executed in a matter of a few months.

The phenotypes of the hits included monopoles, multipoles, long spindles, short spindles, dim microtubules, chromosome misalignment, and pole detachment. A group of RNAi hits backed up Vale’s earlier claims that microtubule nucleation occurs not just at the spindle poles but also within the spindle itself.

Vale sees the screen as a success, especially given that “the spindle is a very well-trodden area,” he said. “This idea that there is a vast sea of unknowns out there is probably unrealistic,” he continued. “However, one should not expect hundreds of hits or even dozens of hits. That is too much to deal with anyway. Even if you can identify just a few gems...that is a very successful outcome.”

References: Glory, E., and R.F. Murphy. 2007. Dev. Cell. 12:7–16.
Goshima, G., and R. Vale. 2003. J. Cell Biol. 162:1003–1016.
Mahoney, N.M., et al. 2006. Curr. Biol. 16:564–569.
Making ductal trees

Vessels and ducts fill a tissue volume using tree-like structures. Building these structures requires not a complex prepattern of attractants and repellents, said Celeste Nelson and Mina Bissell (Lawrence Berkeley National Laboratory, Berkeley, CA), but something far simpler. They reported that the geometry of an existing branching structure directly determines the sites of new branching.

“There is a big void in terms of understanding how these patterns form—why certain cells decide to branch out,” said Nelson. She started by micropatterning a collagen gel with an elastomeric stamp, and then embedding mammary epithelial cells in the resulting cavities. The first patterns were simple bar shapes. Prompted by EGF, which was added uniformly, the cells sprouted only from the ends of the bars. Cells also sprouted from the three termini of a Y-shaped structure, and from the convex face of a bent tubule. All these patterns mimic those seen in vivo, suggesting that cells surrounding the tubules in vivo are not needed to determine basic sprouting patterns.

The various sprouting sites could be predicted by a simple model in which all tubule cells secrete a sprouting inhibitor. This inhibitor falls to local minima at the predicted (and actual) sprouting sites, where it is overcome by the uniform activity of the EGF.

A gradient of the outgrowth inhibitor TGF\(\beta\)1 was present in the predicted pattern. Branching was prevented by excess TGF\(\beta\)1 and induced everywhere by inhibition of TGF\(\beta\)1.

Tubule structures started to inhibit the branching of a neighbor when they were brought to within \(~75\mu\text{m}\)—approximately the spacing seen between neighboring ducts in mouse mammary glands. This inhibition mechanism should maintain the open architecture of the mouse mammary gland.

The new model is simple, but it may not be sufficient. Nelson saw that sprouting increased with increasing tubule length, whereas the simple model predicts either no increase or a decrease. Tension is one possible modifier of the inhibitor model, with tension between the cell mass and the surrounding substrate further encouraging outgrowth. **WW**

Reference: Nelson, C.M., et al. 2006. Science. 314:298–300.

Tubules make nuclear envelopes

The nuclear envelope (NE) retreats into the ER during mitosis and then reemerges during envelope reformation, according to findings presented by Martin Hetzer (Salk Institute, La Jolla, CA).

The model isn’t new but is fighting for acceptance. Those interested in the dynamics of the NE during open mitosis have held onto two models over the years: either the envelope vesiculates before division, or it is resorbed into the ER.

The former theory was quickly and widely accepted when EM data revealed vesicles on the surface of chromatin before envelope reformation. The purification of vesicles with NE markers that distinguished them from ER vesicles supported the argument. The fact that vesicles would be easy to divvy up between daughter cells was a bonus. Many scientists thus believed that the NE reformed after mitosis via fusion of these vesicles.

Still, some researchers worried that the vesicles in the EM images and biochemical experiments might have been created during sample preparation. And when the ER was shown to remain intact during mitosis, the NE field reconsidered its “everything vesiculates” hypothesis.

Hetzer is now firmly entrenched in the resorption camp. Using a novel in vitro assembly system, he showed that NE assembly is more efficient when the membrane fraction is first preformed into an ER-like tubular network. Videos revealed tubules reaching down, tip first, to contact chromatin and then aligning lengthwise. Gaps between tubules were filled by a rapid expansion of the NE, fed by the tubular membrane. Inhibiting prototypical vesicle fusion did not interfere with NE formation.

The vesicle fusion model posed one logistical problem that tubules bypass: how the volume inside two fused vesicles is flattened out into the shape of the envelope. Tubules make it easy—extra volume between the two bilayers can be squeezed back into the ER, or extra lipids can be pumped in from the ER. **NL**

Reference: Hetzer, M.W., et al. 2005. Annu. Rev. Cell Dev. Biol. 21:347–380.

Reference: Hetzer, M.W., et al. 2005. Annu. Rev. Cell Dev. Biol. 21:347–380.

Ductal outgrowth (top) occurs where an inhibitor falls to local minima (bottom).
Moving cells wobble forward, said Erin Barnhart and Julie Theriot (Stanford University, Stanford, CA). The oscillating pathway makes the cells look like a person stepping first on one leg and then the other.

Cell movement is generally studied as either protrusion at the front of the cell or release of adhesions at the rear of the cell. Few if any researchers have concentrated on the links between front and back. Barnhart thinks she has seen signs of this link. She observed that fish keratocytes retracted their trailing edges on first one side and then the other. The result was an oscillation of the lengths of each side of the cell and a wavy path of the cell centroid. These cell strides are signs that the front and rear of the cell are connected by a flexible linker, said Barnhart. When the tension becomes too great for the adhesions at the rear to resist, the adhesions give way.

Simultaneous release by both sides is probably unstable and subject to disruption by any noise in the system, suggests Barnhart. This leaves the cell to release first one side and then the other, in a more stable out-of-phase pattern. She is now concentrating on mathematical modeling and looking for the waddling in other cell types.

Reference: Wilson, C.A., and J.A. Theriot. 2006. *IEEE Trans. Image Process.* 15:1939–1951.

Intercellular communication usually relies on secreted messenger proteins or simple binding events between transmembrane proteins. But Jennifer Gillette and Jennifer Lippincott-Schwartz (NIH, Bethesda, MD) suggested that hematopoietic stem cells (HSCs) and their supporting osteoblasts might talk to each other via an intriguing intercellular transfer of membrane and associated proteins.

HSCs are nurtured by osteoblasts. Gillette wanted to watch the two-cell types interacting, so she labeled some HSCs with quantum dots. “Her first reaction was that it was a bust,” said Lippincott-Schwartz. Many of the quantum dots had ended up on the cocultured osteoblasts.

But there had been no mistake with the labeling. Instead, the HSCs were crawling on the outside of osteoblasts and depositing quantum dots on them. Transfer also occurred with liver cells, which are happy interacting with HSCs, but much less with other cell types. More physiological molecules were also transferred, including a lipid called lissamine that is found in signaling exosomes.

The extracellular release of exosomes normally occurs when multivesicular bodies fuse with the plasma membrane. Alternatively, some viruses can induce the exosomal machinery to form exported vesicles directly from the plasma membrane. The NIH team does not yet know whether either or both of these mechanisms is operating in the HSCs.

Intercellular transfer of molecules has been seen in other contexts. Notch and Delta are two transmembrane proteins that are expressed on different cells; after binding, cleavage, and endocytosis they end up together in one cell. But it is the other section of the cleaved Notch, operating by itself in the other cell, that is the active signaling participant.

The other example occurs in T cells. The T cell receptor binds MHC and bound antigen and draws the whole complex into the T cell. Although this does down-regulate attachment, any downstream signaling is induced by binding rather than internalization.

The NIH team is most intrigued by the possibility that, in their system, signaling may occur downstream of intercellular transfer. Consistent with this theory, the lissamine lipid ended up in signaling-competent endosomes within the osteoblasts.

The downstream consequences of this are, however, unknown. The HSCs may be shedding molecules so that they can detach from the osteoblasts and differentiate; the osteoblasts may be gaining molecules that tell them to recruit and support new HSCs. Gillette has several interventions that should allow her to interrupt the intercellular transfer and thus test its function.

Reference: Février, B., and G. Raposo. 2004. *Curr. Opin. Cell Biol.* 16:415–421.

Bipolar cell movement

Moving cells wobble forward, said Erin Barnhart and Julie Theriot (Stanford University, Stanford, CA). The oscillating pathway makes the cells look like a person stepping first on one leg and then the other.

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Controlling nuclear size

Cells appear to measure and control the size of organelles, but the underlying mechanisms remain mysterious. Frank Neumann and Paul Nurse (Rockefeller University, New York, NY) reported that the size of the fission yeast nucleus can be manipulated by changing the amount of surrounding cytoplasm. The system should allow a genetic investigation of nuclear and cell size control.

As early as 1901, researchers publishing in German noted a fixed ratio between the volume of the nucleoplasm and cytoplasm (the nucleocytoplasmic [N/C] ratio). Various developmental transitions also appear to take their cue from this ratio. In frog embryos, for example, a few nuclei in a large cytoplasm divide rapidly. Only once the N/C ratio increases to a critical level does the organism transition to a more measured cell division schedule.

Neumann found that in fission yeast the N/C ratio was constant under a variety of conditions: throughout the cell cycle; in cells grown under very different conditions; in cell size mutants; and in cells with widely varying DNA content.

When Neumann centrifuged multinucleate cells, he could pack several nuclei into a small amount of cytoplasm. These nuclei grew slowly, whereas nuclei in the same cell that were surrounded by more cytoplasmic space could grow much faster.

It is not clear whether cell size controls nuclear size or vice versa. A clue to the molecular mechanism is also lacking, although nucleocytoplasmic transport might allow the two compartments to communicate information about their relative volumes. With fission yeast, Neumann should be able to address these problems using the power of genetics.

Reference: Umen, J.G. 2005. Curr. Opin. Cell Biol. 17:435–441.

Migrating toward death

Run, kill, and die—these commands from caspase-11 sound like those of an army sergeant ordering the troops to battle. This death- and inflammation-inducing caspase also encourages cell migration, said Juying Li and Junying Yuan (Harvard Medical School, Boston, MA).

The caspases are apoptotic proteases that are conserved from worm to man. The worm situation is simple: caspases focus on death. But mammals are more complex; they express many more caspases and integrate them into other physiological pathways. Li’s example of such integration is caspase-11.

In mice, caspase-11 is induced by bacterial factors and stimulates the release of inflammatory cytokines and the activation of the death effector caspase-1. Direct substrates have been elusive, however, so Li went fishing for caspase-11 binding factors. She found Aip1, an actin-binding protein.

Aip1 activates coflin, which promotes actin destabilization. Li showed that Aip1 activity is increased by caspase-11. Both caspase-11 and Aip1 were necessary for the actin dynamics that drives cell migration. Immune cells lacking caspase-11 were slower to migrate in vitro and failed to home properly to organs in mice.

How caspase-11 times its three commands—migration, inflammation, and apoptosis—is not clear. In the right order, however, this one caspase might drive immune cells to reach an infection site, synthesize antibacterial factors, and commit suicide before inflammation gets out of hand.

Reference: Li, J., et al. 2007. Nat. Cell Biol. 10.1038/ncb1541.
DNA-induced cortical polarity

Chromatin in meiotic cells uses Ran to tell the cell cortex where to establish polarity, said Rong Li (Stowers Institute, Kansas City, MO). Cell polarity in mitotic cells is cued mostly by external information, including contacts with the matrix or with neighboring cells. But for meiotic cells, the signal for polarization comes from their own chromosomes, said Li. The migration of chromosomes toward the cortex helps initiate the extrusion of the polar body, which contains the extra chromosome set.

Li showed that beads coated with DNA added to oocytes induced ectopic spots of contractile cortex, including an actomyosin cap, which extrudes the polar body. The DNA didn’t have to touch the cortex; it induced polarity from up to \(10^{-20}\) \(\mu m\) away. Microtubules and actin were not bridging this distance, as their depolymerization did not prevent formation of the myosin cap, so Li and colleagues considered Ran.

Ran is known to concentrate at meiotic chromatin, and Ran-GTP gradients help drive spindle formation and nuclear envelope assembly. Li showed that Ran also drives cortical polarity in oocytes. Interfering with the activation of Ran blocked the ability of added DNA to create cortical polarity.

With its short diffusion distance (\(~10–20\) \(\mu m\) from the chromatin), Ran-GTP presumably creates only a localized contractile cap, thereby preventing too much cortex and cytoplasm from being lost during extrusion. The cap size depended on the number of DNA beads, which may tailor the size of the polar body according to the amount of DNA to be discarded.

Oocyte cortical polarity is known to require MOS, an upstream kinase of the MAPK cascade in maturing oocytes. Ran seems to concentrate active MAPK around the chromatin, where it might locally activate myosin light chain kinase, which stimulates myosin cortical assembly.

Meiotic chromatin was also recently noted by others to drive a gradient of another GTPase, Rac-GTP, which helps to stabilize the spindle and anchor it to the cortex. Inhibition of Rac activity caused spindle detachment and prevented polar body extrusion. Whether chromatin also induces a contractile cortex in somatic cells to help them round up before mitosis is unknown.

References: Deng, M. et al. 2007. Dev. Cell. 12:301–308. Halet, G., and J. Carroll. 2007. Dev. Cell. 12:309–317.

Lonely DCs mature

Isolation makes dendritic cells (DCs) mature, according to a talk by Aimin Jiang (Yale University, New Haven, CT). The newly discovered maturation pathway may help prevent autoimmunity.

Differentiated DCs cluster together in culture through homotypic interactions of E-cadherin, an adhesion protein that is more typically associated with epithelial cell junctions. Jiang, working with Yale’s Ira Mellman, found that disruption of these cell clusters caused DCs to activate and develop into mature cells capable of migrating to lymph nodes and presenting antigen to T cells. Maturation was previously thought to occur only following the delivery of microbial stimuli.

Loss of adhesion may lead to maturation by releasing \(\beta\)-catenin from junctions so that it can travel to the nucleus, where it activates differentiation-inducing transcriptional programs. Indeed, Jiang showed that overexpression or stabilization of \(\beta\)-catenin caused DC maturation.

Unlike DCs that mature in response to pathogenic factors, those that matured via lost contact did not make inflammatory cytokines. They thus did not induce the strong immunogenic T cell responses in mice that bacteria-exposed DCs did.

DCs constantly leave tissues such as the skin, lung, and intestine, even when no invaders are present. Loss of their previous contacts might activate this new maturation pathway and send the DCs to lymph nodes. Since they lack inflammatory cytokines and probably carry self-antigens, these cells are good candidates for disabling potentially autoreactive T cells.

Reference: Trombetta, E.S., and I. Mellman. 2005. Annu. Rev. Immunol. 23:975–1028.
RNA at the spindle

When Mike Blower started his project on mitotic spindle formation, he never would have guessed he’d end up studying RNA. Now, as head of his own lab at Harvard University (Boston, MA), Blower is elbow-deep in spindle-associated RNAs and is chiseling down their functions.

Mitosis was the focus of his research in Rebecca Heald’s and Karsten Weis’s laboratories at the University of California, Berkeley, CA. There, Blower discovered that a complex of protein and RNA was necessary for spindle assembly. One of the proteins in this complex is Rae1, which during interphase helps to export RNAs from the nucleus. Rae1 needed RNAs to stabilize microtubules and thereby build the spindle.

Blower has now purified and identified many of the RNAs that are associated with microtubule asters. mRNA classes that were enriched on asters included those that encode mitotic regulators, DNA replication factors, and transcription factors needed for developmental patterning. Similar sets of RNAs were found in both frog and human cell extracts.

Blower’s previous work had shown that the RNAs themselves, not their translated protein products, were necessary for spindle assembly. But mRNAs also get translated locally at spindles, as they do at synapses. In his talk, Blower showed that polysomes associated with the spindle were translating the replication and mitosis regulators. Blocking translation during mitosis in cell extracts and in synchronized human cells caused subtle defects in chromosome condensation and alignment, although spindles themselves seemed to form normally.

Local translation of mRNA at the spindle may be simply an efficient way to get mitotic regulators to where they are needed. It might be especially important in oocytes, in which the spindle occupies only a tiny portion of the cell volume.

The function of untranslated mRNAs is a little more difficult to understand. Blower supposes that some of the RNAs, particularly those encoding developmental regulators, attach themselves to the spindle as a way to segregate evenly—or unevenly, in asymmetrically dividing cells—to the daughters. But just how the Rae1 partners help to stabilize microtubules is still unclear.

Reference: Blower, M.D., et al. 2005. Cell. 121:223–234.

Silencing X from a distance

A methyltransferase silences worm X chromosomes from a distance, said Susan Strome (Indiana University, Bloomington, IN). By binding autosomes, this protein may prevent X chromosome silencing proteins from being distracted from their intended targets.

In mammals, females silence one of their two X chromosomes so that their X gene expression levels match that of males. In the worm germline, however, every copy of the X chromosome—two in hermaphrodites and one in males—is silenced. Why worms work this way is unclear; compacting the male X chromosome, which has no pairing partner, into heterochromatin might be necessary for it to segregate intact into a daughter cell during division. Hermaphrodites may silence their two Xs to match the single silent X in males.

Failure to silence the X in germlines makes worms sterile, as revealed by a genetic screen for maternal effect sterile mutants done previously in Strome’s lab.

Several of the proteins that were identified in the screen—MES-2, MES-3, and MES-6—form a complex that binds preferentially to the X chromosome, methylates lysine 27 of histone H3, and thereby silences this chromosome.

In her talk, Strome discussed another sterility protein, MES-4, which her group recently found methylates lysine 36 of histone H3 on the autosomes of germ-line cells. MES-4 avoids nearly all of the X chromosome (due to the presence of MES-2/3/6), yet Strome found it is nonetheless needed to silence X chromosome loci. Microarray analysis showed that loss of MES-4 desilenced ~60 X-linked genes in the germline. Very few autosomal genes were affected.

Strome’s next task will be to determine how MES-4 works from a distance. MES-4 might help the nucleus “gain specificity by preventing promiscuity,” as van Leeuwen and Gottschling proposed for other histone modifiers (Curr. Opin. Cell Biol. 2002. 14:756–762). The presence of MES-4 or its methyl mark on autosomes might repel a direct repressor. If the repressor is present in limiting amounts, it would be titrated away by the more abundant autosomes in the absence of MES-4 and thus fail to silence the X chromosomes. This limiting repressor is probably not the MES-2/3/6 complex, as its methylation patterns are unaffected by the loss of MES-4.

Reference: Bender, L.B., et al. 2006. Development. 133:3907–3917.
**Tension-induced tumors**

Increased tissue stiffness can pull premalignant cells into invasion and tumorigenesis, said Kandice Johnson and Valerie Weaver (University of California, San Francisco).

Amongst biologists seeking tumor promoters, mechanical forces have attracted less attention than individual gene products. Weaver has for some time wanted to correct this imbalance. At her former lab at the University of Pennsylvania, she was surrounded by bioengineers interested in mechanics and physical principles. “I was hearing this stuff day in and day out,” she said. “After a while, you start to think differently.”

Weaver’s first focus was tumors. Stiffer tumor lesions have been associated with poorer prognoses. In vitro, tension leads to various changes in two-dimensional cultures, but only recently has Weaver’s group tested the effect of tension on three-dimensional cultures of mammary epithelial cells.

They found that adding more collagen to increase matrix rigidity destroyed tissue organization: lumen formation was inhibited; and cell division increased. Matrix stiffness destabilized the cell–cell linkages of adherens junctions but promoted the cell–matrix links of focal adhesions and their associated pro-division signaling. In normal tissues, such forces may guide cell growth during development and direct cells into stiff wound tissue.

To switch the focus to whole animals, Weaver lab members Johnson and Laura Kass used mice overexpressing the Her2/neu oncogene. They found that stiffness of the tumor and surrounding stroma increased during tumorigenesis and was higher than normal even in premalignant tissue. The collagen fibers became linearized and taut, suggesting that the organization and tension of fibers may be as important as their quantity.

Tension can also be increased by cross-linking. Fibroblasts expressing the cross-linking protein lysyl oxidase promoted the growth and invasiveness of ras-expressing premalignant cells. Weaver’s group now has preliminary results that lysyl oxidase production may be turned on in certain tumor cells and inhibiting it may restrict tumor formation in an animal model. **WW**

Reference: Paszek, M.J., et al. 2007. Cancer Cell. 8:241–254.

**Keeping an attractant within limits**

For man and yeast alike, the stench of too much perfume can kill a dating opportunity. Matthieu Peil and Andrew Murray (Harvard University, Cambridge, MA) presented evidence that the Bar1 protease keeps the attractant α factor to a level that a partner yeast cell can interpret. Only in this narrow concentration range can the prospective partner polarize correctly and choose between equally attractive mates.

This yeast strategy differs from that used by chemotactic cells, which can sense signals of widely varying intensity by adjusting the affinity and response of their receptors. These cells are seeking, and moving toward, targets that are far away.

By contrast, mating yeast, neuronal growth cones, and pollen tubes are seeking to contact or fuse with cells that they can reach without moving their cell bodies. The proteolysis solution used by yeast may be common to several such systems, suggested Piel and Murray, as these systems share evidence of both protease involvement and narrow detection ranges.

In budding yeast mating, Bar1 is the protease of interest. It is part of a simple attraction system: α cells make α factor to attract a cells, their mating partners. The a cells need Bar1 to degrade α factor because α factor concentrations rise with a cell number and concentration.

Piel and Murray used flow chambers to show that yeast lacking Bar1 respond effectively to only a narrow range of concentrations of α factor. In mating mixes, adding more α cells reduced mating efficiency unless the a cells could make Bar1. High levels of α factor induced production of Bar1, which reduced the concentration of surface α factor to a moderate level (as measured by a transcriptional readout).

Cells lacking Bar1 performed better if protease was supplied exogenously, but this did not compensate entirely. Only the cells with endogenous Bar1 could efficiently pick between two identical partners. Modeling suggests that Bar1 targeted to the polarized cell surface helps distinguish between a single concentrated signal and two overlapping signals. **WWW**

References: Barkai, N., et al. 1998. Nature. 396:422–442. Rosoff, W.J., et al. 2004. Nat. Neurosci. 7:678–682.