Citations

Field Work

Magnetic fields have done much for biology, from magnetic resonance imaging to micromanipulation of cells. Now, in an article in *Nature Nanotechnology*, Souza et al. report that magnets might replace rotational flasks or gel-based matrices for 3-D cell culture. As even the most cursory examination of any multicellular organism will show, the native environment of cells is one surrounded by other cells and structural features, so moving beyond traditional 2-D culture would enhance the fidelity of in vitro systems in mimicking the complex intercellular interactions and functional roles found in vivo. In Souza et al.'s strategy, the cells are made responsive to a magnetic field by incubating them with dispersed fragments of a hydrogel formed from gold nanoparticles, magnetic iron oxide nanoparticles, and phage particles displaying an integrin-binding peptide. The hydrogel fragments bind to the cell and can undergo integrin receptor-mediated internalization, so when a magnet is placed above the culture vessel, the cells are levitated within the medium. Since the magnetic force is not sufficient to overcome the surface tension of the medium, the cells accumulate at the liquid-air interface, where they gather directly under the magnet and form spherical multicellular structures. Compared to cells in 2-D culture, magnetically levitated human glioblastoma cells did not require passaging and grew faster. Based on expression of N-cadherin, a marker of intercellular contact, the levitated cells also more closely resembled cells growing in the in vivo model system of human tumor xenografts in immunodeficient mice. Preliminary studies also showed that the multicellular structure could be influenced by the shape of the magnet, while experiments in which separately cultured glioblastoma cells

Magnetic fields, such as illustrated above, are enabling 3-D culture of cells made magnetically responsive through incubation with iron oxide and gold nanoparticles.

and astrocytes were later combined showed the feasibility of studying interactions of different cell types brought together by magnetic guidance. Compared with scaffolds made of biodegradable material, magnetic levitation offers simpler setup and much faster propagation. Moreover, the magnetic hydrogels can easily be adapted to incorporate phage bearing other peptide ligands and—unlike some 3-D culture methods—allow use of standard culture medium. The new tissue culture system is expected to have applications in bioengineering, stem cell research, and biotechnology.

Souza et al. Three-dimensional tissue culture based on magnetic cell levitation. *Nat. Nanotech.* [Epub ahead of print, March 14, 2010; doi: 10.1038/NNANO.2010.23].

Make the Switch

Different flavors of fluorescent highlighter proteins respond to light by switching on, changing emission wavelength, or toggling their fluorescence on and off in response to particular illumination triggers. Unlike static versions, such as GFP, highlighters are perfect for pulse labeling, making them ideal for visualizing protein trafficking processes. Photoconvertible varieties of highlighters, which are fluorescent before and after a light trigger, are particularly popular because they simplify cell tracking. However, current photoconvertible fluorescent proteins can be hamstrung by inefficiency, rapid bleaching, or the need for multimerization. In the *Journal of Biological Chemistry*, Welman et al. offer a new tool, photoactivatable Green Cherry (G^{PAC}), which is a fusion of the red fluorescent protein (RFP) monomeric Cherry with

a photoactivatable variant of GFP. The fusion protein is designed to offer uninterrupted red fluorescence to designate the marker-expressing cells, while green fluorescence should remain nonexistent until triggered by a burst of 405-nm light. As intended, G^{PAC} -expressing cells showed strong red signal before and after photoactivation, and green fluorescence was trigger-dependent and persisted for a few hours. In several tagged proteins tested, G^{PAC} tolerated fusion proteins positioned at its N or C terminus without losing activity or noticeably affecting localization or function of the cellular protein, even when the fusion partner required extensive posttranslational processing and modification such as myristoylation or farnesylation. The authors show the application of G^{PAC} in investigating cytoskeleton dynamics in cultured cells, and in studying migration of immune cells in vivo in *Drosophila*. Such in vivo applica-

tions are likely to be a particular advantage of the G^{PAC} strategy, which—unlike the recently described photoconvertible protein Pharmet—does not rely on cyan fluorescence or fluorescence resonance energy transfer (FRET), both of which can suffer from limited range in tissue. One potential stumbling block for G^{PAC} is the relatively large size of the fluorescent tag (>50 kDa); this feature requires G^{PAC} -fused proteins to be carefully validated before using in functional studies. Nevertheless, G^{PAC} offers a convenient means to follow tag-expressing cells or subcellular compartments (via red fluorescence) while highlighting a photoconverted subset (green signal) in ongoing monitoring of spatiotemporal dynamics.

Welman et al. 2010. Two-color photoactivatable probe for selective tracking of proteins and cells. *J. Biol. Chem.* 285(15):11607-11616.

It's FLPing better!

Site-specific recombinases (SSRs) have proven to be indispensable tools for genetic engineering of mammalian cells. This is especially true in the case of conditional gene targeting experiments where the precise spatial and temporal activation or inactivation of a transgene is required. Cre and FLP recombinases are the two most commonly used SSRs, often being employed in tandem for more complex applications such as when expression of a specific gene is induced by one SSR, while its subsequent inactivation is induced by another. Dual SSR methods are often hindered, however, by the very poor recombination efficiency of FLP (6%) in comparison to Cre (60%). Even an optimized FLP variant (FLPe) that was derived by molecular evolution has only 25% of the activity of Cre. But in a recent *Stem Cells* paper, Patsch et al. demonstrate that direct delivery of their cell-permeant version of FLP protein into cultured mammalian cells significantly increases recombinase activity. This recombinant FLP is fused to the cell-penetrating peptide Tat, a nuclear localization signal to enhance nuclear uptake, and a His-tag for purification of the bacterially expressed protein. Using a reporter cell line

that activates *lacZ* expression following a FLP-mediated recombination event, the purified "TAT-FLP" protein at a concentration of 3 μ M was capable of inducing half-maximal activation in 30 min, and maximum activation by 3 h. The highest recombination rate, around 75%, was obtained with 4 μ M TAT-FLP. Comparable results were obtained for the equivalent embryonic stem (ES) cell *lacZ* reporter line, and it was demonstrated that TAT-FLP treatment of ES cells did not affect their germline transmission. The authors went on to show that the TAT-FLP protein was effective for reversible transgene induction in a dual recombinase system when used with a cell-permeant version of Cre by demonstrating the efficient reversible expression of a FLP-ON-Cre-OFF-LacZ construct in NIH-3T3 cells and a Cre-ON-FLP-OFF construct of the caudal-related homeobox protein CDX2 in human ES cells. Together, these results demonstrate the robustness and flexibility of the TAT-FLP system.

Patsch et al. Engineering cell-permeant FLP recombinase for tightly controlled inducible and reversible overexpression in embryonic stem cells. *Stem Cells*. [Epub ahead of print, March 23, 2010; doi: 10.1002/stem.417].

PAR-CLIP

Vertebrate genomes encode several hundred RNA-binding proteins (RBPs) that regulate editing, stability, and translation of RNA transcripts. Knowledge of the binding and activity of these protein complexes is integral to understanding the regulation of gene expression at the posttranscriptional level. One method used for identifying stable RNA-RBP interactions is RIP-Chip, which involves the immunopurification of RBPs followed by microarray profiling of bound RNA. While this approach will readily define an RNA target stably interacting with a probed RBP, it does not provide data on the specific recognition sequence or binding site of the RBP and cannot identify transiently or loosely associated RNAs. One solution is to use the crosslinking with immunoprecipitation (CLIP) method, which crosslinks RNA-RBP complexes prior to immunoprecipitation. In these experiments, bound RNAs are isolated, reverse-transcribed, and the resulting cDNAs sequenced. CLIP is limited by low efficiency of the UV crosslinking step, since non-crosslinked RNA molecules are more readily reverse transcribed. This leads to high signal-to-noise ratios and difficulty in distinguishing between the crosslinked and non-crosslinked target RNAs. Building upon this method, Hafner et al. describe photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) in the April 2, 2010 issue of *Cell*. The authors applied 4-thiouridine (4SU), a photoactivatable nucleoside

Diagram of the PAR-CLIP procedure. Reprinted with permission © 2010 Elsevier.

analog, to cultured cells expressing a FLAG/HA-tagged RBP to facilitate crosslinking. Exposure to 365-nm light crosslinks 4SU-containing RNAs to associated RBPs, which were subsequently isolated by immunoprecipitation. Covalently-bound RNA recovered from the complexes was digested with RNase T1, radiolabeled, and separated by electrophoresis. When reverse-transcribed, guanine was specifi-

cally misincorporated opposite the cross-linked 4SU residues, indicating the location of the crosslink with a thymidine-to-cytidine (T-to-C) transition. This allowed the authors to precisely determine the RBP binding site by Illumina sequencing and comparison to sequence databases. Focusing on clusters with high frequencies of T-to-C mutations—rather than sequences with the highest number of reads—significantly improved the ability to separate signal from noise, representing a noteworthy improvement over previously published methods. Another advantage of the new approach is that photoactivatable nucleosides facilitate higher yields of crosslinked RNAs with similar radiation intensities. Data showed that the photoactivatable nucleosides improved RNA recovery 100- to 1000-fold when compared to conventional UV crosslinking at 254 nm. The authors validated PAR-CLIP by determining the transcriptome-wide binding sites of several known RBPs and microRNA-containing ribonucleoprotein complexes at high resolution.

Hafner et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141:129-141.

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