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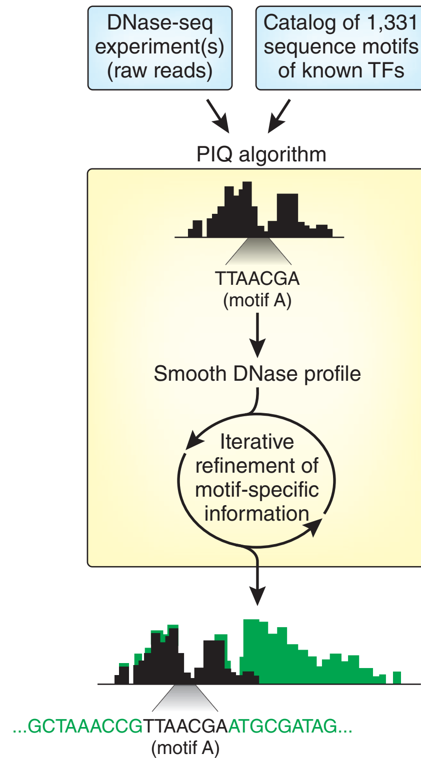

Figure 2 PIQ improves the identification of occupied transcription factor (TF) binding sites *in vivo* from genome-wide DNase I hypersensitivity data. The input for PIQ is one or more DNase-seq experiments and 1,331 transcription factor motifs represented as position-weight matrices. First, PIQ scans a DNase profile to determine whether each individual motif is located at a relative gap in DNase hypersensitivity, indicating that a protein occupies the site. A unique signature profile is shown for a hypothetical motif A (black). Second, raw reads are smoothed, by modeling them as arising from a Gaussian distribution, over each experiment to increase robustness to low coverage and noise. In the last step, estimation of motif-specific information and computer-generated, hypersensitivity-profile building steps are iteratively performed to calculate the probability that each motif is bound. Motif-specific information about the expected DNase profile surrounding a bound site allows for deciphering of complex profiles at enhancers and promoters. As a hypothetical example, motif A (black) is shown to be responsible for the left half of the total DNase profile (green) of a putative enhancer subregion. Integration of time-series data by PIQ can elucidate hierarchical and directional sequences of transcription factor binding.

the development of efficient cell replacement or regeneration therapies for human disease. Current *in vitro* protocols attempting to make large numbers of pancreatic beta cells from human embryonic stem cells mostly induce a slightly incorrect, off-track developmental program[8]. Does mismanagement of pioneer factor–dependent changes in chromatin architecture prevent entry into or failure to maintain a proper differentiation track? As modeled in **Figure 1d**, inefficient entrainment might reflect inappropriate corepressor recruitment by pioneer factors[9]. Deficiencies in the first rounds of settler-migrant binding (either absence or a reduced rate) could obstruct a population-wide change toward the next stage of cell differentiation. Thus, PIQ may identify novel transcriptional manipulations to increase differentiation toward the desired cell lineages.

A linked notion is that scoring by PIQ analysis could provide a deeper assurance that differentiation protocols are moving along correctly. It will be interesting to see to what degree PIQ analyses can be carried out on differentiating cell populations captured from a natural *in vivo* context. Although its robustness to noise implies that PIQ could help analyze DNase-seq data from smaller numbers cells, experiments to define its lower limit of sensitivity should be extremely useful.

PIQ is limited in that it calculates DNase footprints only for single transcription factors rather than explicitly performing a joint modeling of footprints and DNase profiles for multiple transcription factors. Moreover, Sherwood *et al.*[1] did not use PIQ to address if and how transcription factors are capable of mediating chromatin repression. Somewhat conspicuously, they did not identify Foxa1 as a pioneer factor, even though it is one of the few thoroughly validated pioneers[2]. They venture that this 'miss' is either explained by the cell type assayed or because Foxa1 may have both chromatin opening and compacting activity.

The results of Sherwood *et al.*[1] suggest that PIQ is an important new tool for understanding how transcription factor expression and chromatin architecture are integrated at a functional level. It should be useful for assessing how histone modifications and other regulatory mechanisms, such as DNA methylation and passive forms of cooperativity between transcription factors[2,7], enhance or restrict pioneer factor binding *in vivo*. There seems to be no reason why PIQ could not be equally applicable to animal or plant tissues, and to gain insight into the dynamic remodeling of chromatin not only during embryogenesis, organogenesis and normal tissue maintenance but also during the abnormal processes that occur in metaplasia and cancer.

COMPETING FINANCIAL INTERESTS
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Of snowflakes and natural killer cell subsets

Lewis L Lanier

Mass cytometric analysis reveals a remarkable and unanticipated degree of phenotypic heterogeneity among human peripheral blood natural killer cells.

In the beginning, natural killer (NK) cells were often referred to as 'null' cells because they were thought not to express any defining cell surface markers that could be used to identify and distinguish them from other classes of leukocytes, and because their origins and relationships to other hematopoietic cells were not understood. In a recent issue of *Science Translational Medicine*, Blish and colleagues[1] definitively render this 'null' moniker null and void by revealing the existence of more than 6,000–30,000 phenotypically distinct NK cell subsets in the blood of any single human. Although it will be challenging to elucidate the functional and physiological meaning of each of these NK cell subsets, the appreciation that such extensive heterogeneity exists within the NK cell lineage implies that this diversity may have evolved to deal with specific pathogens and to detect transformed or stressed host cells.

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Figure 1 Subsets of subsets of subsets. Leukocyte subsets are defined and enumerated by first classifying cells into a distinct lineage using informative positive and negative markers, for example, for human NK cells, lack of cell surface CD3 and expression of CD56. Then, using reagents that detect molecules that are heterogeneously expressed within the lineage (e.g., CD94, CD16), it is possible to further describe subsets within the population, an exercise much like opening nested Russian dolls. Using a sufficient panel of markers, the ultimate 'subset' may be one cell, in that even within the clonal progeny of a lymphocyte, each cell may display a unique array of proteins, lipids, carbohydrates and nucleic acids.

Blish and colleagues[1] arrived at these conclusions thanks to recent technological advances in mass cytometry. This approach enables simultaneous analysis of unprecedented numbers of cell surface markers. The quest to identify and sort lymphocyte subsets based on the simultaneous detection of two cell surface proteins began in 1977 when Len Herzenberg (the father of fluorescence-activated cell sorting (FACS) technology) and colleagues used the excitation of fluorescein- and rhodamine-conjugated polyclonal antisera to distinguish subsets of mouse B cells defined by single or dual expression of IgM and IgD[2]. Seven years later, Herzenberg developed two-laser FACS instruments, which allowed further refinement of B-cell subsets based on simultaneous 3-color immunofluorescence[3] and the first analysis of the complexity of human NK cell populations[4]. Thanks to continued technological advancements, today 18-color, 20-parameter flow cytometry is possible, and 9- and 10-color flow cytometry is routine in many laboratories[5].

Even so, as noted in Herzenberg's original 1977 article, a major limitation of fluorescence-based methods is the spectral overlap between the fluorescent dyes. This results in compromises; for example, because spectral emissions of fluorochromes are often broad, to minimize overlap with the emissions of other fluorochromes excited by the same light source one is often forced to collect only a fraction of the emitted light. Another limitation of fluorescence-based analysis is that even unlabeled cells emit autofluorescence, thus reducing the signal-to-noise ratio.

In the present paper, Blish and colleagues[1] analyzed expression of a larger panel of NK cell surface markers than would be possible with flow cytometry through the use of mass cytometry, a method recently used to reveal diversity among human bone marrow cells[6] and $CD8^+$ cytotoxic T lymphocytes[7]. This approach overcomes the limitations of fluorescence-based methods by conjugating detection reagents—typically monoclonal antibodies—to rare metals rather than to fluorochromes. Because these metals do not exist in hematopoietic cells, there are no background emissions like autofluorescence. In addition, due to the unique mass of each metal, one circumvents the need for compensation (unlike fluorophores, which have emission spectra that can overlap). Rather than being excited by lasers (as in FACS), cells labeled with rare metal-conjugated reagents are vaporized and the mass of the metal bound to the cell is quantified by mass spectrometry using an instrument referred to as a CyTOF (cytometry by time of flight).

Blish and colleagues[1] simultaneously analyzed 37 parameters (using reagents that exclude dead cells or cells of other lineages, and monoclonal antibodies that define 28 NK cell surface receptors) on NK cells from the peripheral blood of 22 healthy adults, including five sets of monozygotic twins[1]. The use of adult twins provided the opportunity to determine whether components of the NK cell phenotype are dictated by genetics or the environment. An interesting insight resulting from the study was that whereas the expression of inhibitory receptors (e.g., inhibitory killer immune receptors) was largely genetically determined, many of the activating receptors differed in expression between twins, suggesting that expression of these receptors is shaped by environmental factors such as current or past exposure to pathogens.

But perhaps the most remarkable finding from this study was the extent of heterogeneity observed among NK cells. For example, even the most abundant subset of NK cells within an individual comprised no more than 7% of the total NK cell population, and collectively the 50 most prevalent subsets accounted for only about 15% of the total NK cell population. Knowing that human blood contains ~300–400 NK cells per microliter[8], the authors calculated that they analyzed only 3,500 to 35,000 NK cells per donor. Assuming that this relatively limited analysis may have overlooked more rare NK cell subsets, the authors used several established ecological methods to estimate the total number of different NK cell subsets present in all individuals in this cohort (108,000–125,000) and in any one individual (6,000–30,000).

Furthermore, this study may, if anything, underestimate the heterogeneity among NK cells. For example, if cells are classified as positive or negative for a given marker (as done by Blish and colleagues[1]), by examining 28 cell surface markers it is theoretically possible to define 268,435,456 combinations of those markers. That said, cell subsets are often classified as 'bright', 'intermediate', or 'dim' for expression of a given marker; incorporating these more subtle gradations of marker expression will likely dramatically increase the number of subsets identified. Furthermore, NK cells in bone marrow, spleen, lymph nodes, lung, liver and decidual tissues are known to be phenotypically different from peripheral blood NK cells. Analyzing NK cells from different tissues may result in multiplicative increases in the total number of NK cell subsets identified. In other words, the findings of Blish and colleagues[1] may have revealed only the tip of the iceberg of diversity within the human NK cell lineage.

A great deal of additional work will be needed to elucidate the biological basis of each observed phenotypically defined NK cell subset. For example, like other hematopoietic cells NK cells arise from a single stem cell that undergoes a progressive and nonsynchronous program of differentiation until reaching a mature terminal stage. Also, like other leukocytes, NK cells have the capacity to sense their environment; these cells respond to environmental changes in the presence of growth factors, chemokines, cytokines, and intracellular and extracellular commensal and pathogenic microbes by changing their phenotype. Therefore, it remains to be determined how many of the NK cell phenotypic subsets identified by Blish and colleagues[1] represent NK cells at different differentiation or maturation stages, NK cells exhibiting transient phenotypic variations resulting from the influence of intrinsic or extrinsic stimuli, or functionally distinct, terminally differentiated effector lineages. In addition, this study presents a 'snapshot' of the NK cell population. Longitudinal analysis of healthy and diseased subjects will be needed to distinguish genuine NK cell

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subsets from distinct activation states. Longitudinal analyses will also shed light on the influences of age on the NK cell repertoire, diurnal fluctuations in the NK cell subsets, and responses to therapeutic treatments for infectious disease and cancer.

These intriguing and unexpected results raise the more general question of what constitutes a cell 'subset'. The convention is to first identify a cell by the expression of one defining phenotype—for example, human NK cells can be defined as lymphocytes lacking cell-surface CD3 and expressing CD56—and then progressively narrow the subset by scoring cells as positive or negative for other markers such as CD94, inhibitory killer immune receptors and chemokine receptors. Much like nested Russian dolls (**Fig. 1**), as one adds more markers the subsets get smaller and smaller. However, with advances in CyTOF technology, quantitative measurement of a hundred or more parameters is foreseeable, and these parameters may go beyond the typical analysis of cell-surface proteins to include DNA, RNA (both coding and noncoding), carbohydrates, lipids and post-translational protein modifications. At this point, what really constitutes a subset? Ultimately, like snowflakes, each cell will undoubtedly be unique. The real challenge is to understand the physiological meaning of this remarkable phenotypic diversity and how it relates to the immune function of these cells.

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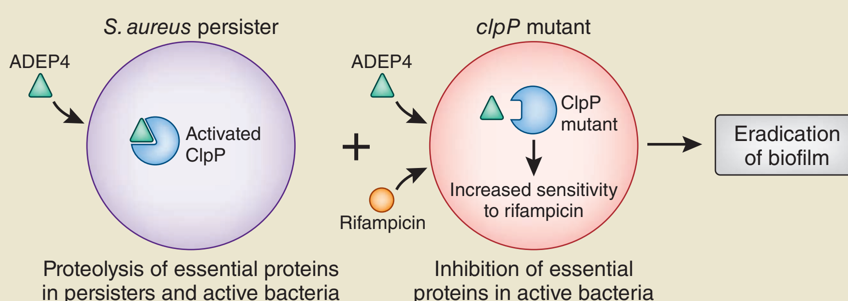
A one-two punch knocks out biofilms

Biofilm formation, a tactic used by some bacterial pathogens to evade drug treatment and the human immune response, is a growing threat to human health. Now, Conlon *et al.*[1] describe a novel strategy to destroy biofilm-associated *Staphylococcus aureus*, a pathogen that can cause a range of severe diseases. They report in *Nature* that two antibiotics, when used together, eliminate *S. aureus* growing in biofilms, kill methicillin-resistant *S. aureus* (MRSA) and eradicate a deep-seated *S. aureus* infection in mice.



The two antibiotics are acyldepsipeptide 4 (ADEP4) and rifampicin. ADEP4 had been shown to kill replicating *S. aureus*[2] but it had not been tested against so-called bacterial persisters, phenotypic variants that tend to grow in biofilms. Because commonly used antibiotics target processes essential to bacterial replication, they are often ineffective against metabolically inactive persisters.

Conlon *et al.*[1] show that, whereas antibiotics such as vancomycin and ciprofloxacin are only marginally effective against *S. aureus* persisters, ADEP4 reduces their numbers exponentially within a day. ADEP4 acts by binding to ClpP, the catalytic core of a bacterial protease, activating it to degrade proteins indiscriminately[2]. A large quantitative proteomics study reveals that ADEP4-ClpP targets more than 400 proteins for degradation, and first among them are essential ribosomal proteins[1]. Conlon *et al.*[1] suggest that the efficacy of ADEP4 against persisters may be linked to its ability to uncouple ClpP from ATP dependency[2], activating the protease in dormant cells that have low energy levels.

"It's really important to find new antibiotics, particularly those that are active against *S. aureus* and against bacteria in biofilms," says Carl Nathan of Weill Cornell Medical College in New York. "The discovery that ADEP4-mediated activation of ClpP is efficacious against *S. aureus* is significant because it represents a way to kill a group of nonreplicating or slowly replicating bacteria by targeting a pathway that is not on the classical list." Conlon *et al.*[1] find that the effects of ADEP4 are impressive but transient, because bacterial growth quickly rebounds as *clpP* mutants arise that are resistant to ADEP4. But *clpP* mutants have lower fitness and are 10–100 times more susceptible to rifampicin compared with wild-type *S. aureus*. So even though rifampicin on its own is ineffective against *S. aureus* persisters, when combined with ADEP4 it reduces *S. aureus* cell counts to extremely low levels.

The problem of antibiotic-resistant biofilms is becoming increasingly acute with the rise in medical interventions such as insertions of catheters and prostheses and the paucity of new antibiotics with unconventional mechanisms of action. The efficacy of ADEP4 and rifampicin against *S. aureus* biofilms and MRSA is a tale of success, but more work is needed to determine whether this approach will work against other biofilm-forming and antibiotic-resistant pathogens. "It would be a big mistake to assume that the word 'biofilm' refers to exactly the same thing no matter what community of organisms is causing it, which anatomical site you find it in or how long it's been there," says Nathan. Similarly, "persistence is an operational definition, and we still don't know whether bacteria that persist against one antibiotic are similar to those that persist against a different antibiotic," Nathan adds.

The principle that bacterial persisters can be killed by activating an inactive target represents a promising alternative to the conventional approach of seeking to inhibit active pathways. A deeper understanding of the processes that are active and inactive in persisters and in biofilm formation will be critical to the identification of additional drug targets. While the hunt for new antibiotics continues, activating ClpP to kill *S. aureus* persisters holds promise for treatment of a dangerous human pathogen.

Irene Jarchum
Locum Assistant Editor

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