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observations have led to a vigorous discussion as some groups argue that they see aberrations less frequently than do others. Whether the different experiences of different laboratories are explained by the different ES lines used, which have diverse starting genotypes, or whether they are due to different subculturing techniques, or culture conditions, or simply 'luck,' remains to be resolved.

Nevertheless, a striking feature of the reported karyotypic changes is that they commonly involve acquisition of extra copies of the same chromosomes, chromosomes 17 and 12. Moreover, extra copies of these chromosomes, or more precisely the long arm of chromosome 17 (17q) and the short arm of chromosome 12 (12p), are almost always found in embryonal carcinoma (EC) cells[6,7], the pluripotent stem cells of teratocarcinomas and the malignant counterparts of ES cells. This commonality of genetic change in ES cells in culture and EC cells in tumors suggests a common cause. An obvious possibility is the selection for variants that affect the molecular decision processes by which a pluripotent stem cell chooses between the alternative fates of death, differentiation or self-renewal; even a small increase in the probability of self-renewal over death or differentiation would provide a strong selective advantage (**Fig. 1a**). Such variant 'selfish' ES cells may indeed behave more like EC cells in teratocarcinomas[2,8].

Currently, we only have fragmentary knowledge of the mechanisms that promote survival and self-renewal of human ES cells. Like murine ES cells, they express the transcription factors Oct4, Sox2 and Nanog. But they also differ from murine ES cells. Leukemia inhibitory factor is unable to support human ES cell growth in the absence of feeders[9], and it is reported that high concentrations of fibroblast growth factor 2 and also transforming growth factor β, activin and nodal, but not the bone morphogenetic proteins, promote human ES cell self-renewal[10,11].

The results reported by Pyle *et al.* and Herszfeld *et al.* provide important new information. First, Pyle *et al.* found that TRKB and TRKC receptors are expressed by human ES cells and, most strikingly, addition to the culture of their ligands, the neurotrophins brain-derived neurotrophic factor, neurotrophin (NT)3 or NT4, raises the notoriously low cloning efficiency of these cells some 36-fold (**Fig. 1a**). Certainly, in our hands, we have only seen such cloning efficiencies in human ES cells that had acquired an abnormal karyotype. Yet in the reported study, the cells remained diploid, and fully pluripotent. In further experiments, Pyle *et al.* showed that the neurotrophins promote ES cell survival and suppress apoptosis through the PI-3-kinase pathway. At least some of the beneficial effects of the mouse embryo fibroblasts commonly used to support human ES cell growth may be due to their ability to produce neurotrophins.

In their study, Herszfeld *et al.* monitored the expression of CD30, a member of the tumor necrosis factor receptor superfamily (**Fig. 1b**). They found that diploid human ES cells do not express CD30, whereas EC cells and several karyotypically abnormal ES cell lines derived from initially diploid lines showed faster population growth rates and all expressed cell surface CD30. These cells also expressed a splice-variant mRNA encoding a truncated form of the CD30 protein consisting of a constitutively active, cytoplasmic domain. This expression of CD30 correlated with an increased resistance to apoptosis, a function that was also demonstrated by transfecting human ES cells with a vector encoding constitutive expression of the cytoplasmic form of CD30; expression of this transgene was associated with a decreased level of apoptosis.

Both papers invoke the importance of controlling survival of ES cells in culture as a factor in optimizing culture conditions for human ES cells. Clearly, neurotrophins are important candidate factors to add to new formulations for defined media for human ES cell culture. Moreover, in any eventual clinical application of ES cell–based regenerative medicine, persisting ES cells that are inadvertently transferred to a patient might be killed using pharmacological inhibitors of the PI3-K pathway. Likewise, the results of Herszfeld *et al.* suggest that monitoring another pathway involving CD30 and NFκB can provide a tool for identifying variant cells in human ES cultures and a means of eliminating them.

Although these two reports now add new important pieces to the jigsaw puzzle of human ES cell growth control, we are still a long way from seeing the whole picture. Pyle *et al.* noted that the neurotrophin NT3 and the neurotrophin receptor $p75^{NGFR}$ are encoded by chromosomes 12 and 17 respectively, so that amplification of either chromosome could enhance neurotrophin signaling and provide a selective advantage. However, $p75^{NGFR}$ expression was not detected in ES cells. In the case of the expression of CD30, Herszfeld *et al.* found a correlation with the appearance of an abnormal karyotype, whether or not the abnormalities affected chromosome 1, which encodes CD30. Although the neurotrophin and CD30 pathways might be ways in which biologists, or the ES cells themselves, can control ES cell survival, it seems likely that other pathways and other genes will also be found to play important roles. Nevertheless, not only do these papers provide insights into the mechanisms driving selection of variant human ES cells, but they also suggest approaches to optimizing culture conditions to reduce their selective advantage, and a means to eliminate variant cells when they appear.


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# Self-illuminating quantum dots light the way

John V Frangioni

**A quantum dot decorated with luciferase molecules fluoresces without external illumination.**

In this issue, So *et al.*[1] describe a clever quantum dot technology that permits improved imaging *in vivo* compared with existing quantum dots. The new probes rely on bioluminescence resonance energy transfer[2], which converts chemical energy into photon energy, resulting in dramatic increases in fluorophore excitation and dramatic reductions in the effects of tissue autofluorescence. The technology has several exciting

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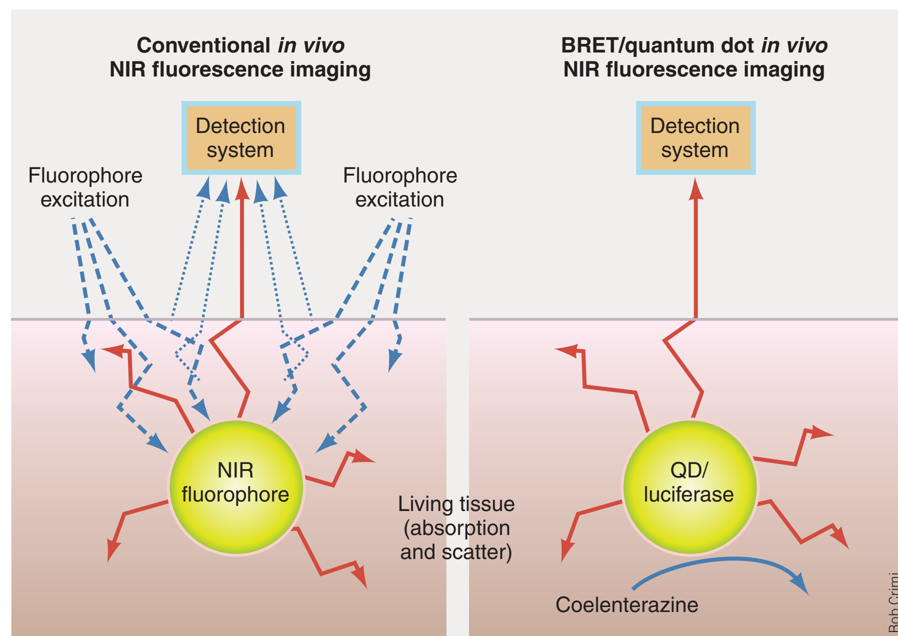


**Figure 1** In conventional near-infrared fluorescence imaging (left), excitation light (dashed lines) is scattered and absorbed by tissue. This results in reflected excitation light and autofluorescence (dotted lines), weighted heavily on the surface, which interferes with the detection of desired fluorescence (solid lines). Using quantum dot/luciferase conjugates described by So *et al.* (right), chemical energy from the substrate coelenterazine is converted into photon energy, which excites the quantum dot through bioluminescence resonance energy transfer (BRET). No excitation light is required, and autofluorescence is virtually eliminated, although emitted photons are still subject to absorption and scatter before detection.

features. It eliminates the need for fluorescent excitation light and takes advantage of quantum dots' blue-increasing extinction coefficient. It greatly reduces the high background caused by surface illumination and tissue autofluorescence and exploits tissue-penetrating near-infrared wavelengths for fluorescence emission. It efficiently couples chemical energy with light energy. And, finally, because energy transfer is resonant, and nonradiative, absorption of excitation photons by hemoglobin and other tissue pigments is virtually eliminated.

Finding fluorescent objects in scattering tissue using light can be quite difficult. The use of near-infrared wavelengths (typically 700–900 nm) for excitation and emission helps to reduce absorption and scatter, and frequency- or time-domain techniques can be used to improve depth sensitivity even further (reviewed in refs. 3–5). However, even in the near-infrared range[6], tissue autofluorescence always remains a major and often overlooked problem that reduces the signal-to-background ratio and, therefore, object detectability (**Fig. 1**).

To improve fluorophore excitation and reduce the effects of tissue autofluorescence, So *et al.* covalently attached multiple molecules of *Renilla reniformis* luciferase to a single fluorescent semiconductor nanocrystal (that is, a quantum dot), forming a large conjugate >22 nm in hydrodynamic diameter. When luciferase binds its substrate coelenterazine, it emits broad-spectrum blue light peaking at 480 nm. So *et al.* show that the blue light emitted by the luciferase molecules in the conjugate excites the bound quantum dot. As the absorption spectrum of quantum dots increases exponentially towards the blue, the complete overlap of the luciferase emission and quantum dot absorption spectra provides extremely efficient fluorophore excitation without the addition of exogenous light.

The authors characterized this system in both *in vitro* and *in vivo* experiments, providing convincing evidence that bioluminescent quantum dots can be spectrally multiplexed, and that they outperform conventional quantum dot imaging. It should be emphasized that the magnitude of improvement of this technology over external excitation is dramatic. As detailed in the paper's online supplementary information, to excite quantum dots embedded 1 cm below scattering tissue equivalently, one would need 9W of 480-nm external excitation light, compared to a single injection of coelenterazine. Tissue autofluorescence from such high levels of excitation light would also be considerable, as would the inevitable excitation-light leakage through emission filters. This helps explain the authors' result that bioluminescent quantum dots embedded in 3 mm of scattering tissue were visible *in vivo* using bioluminescence resonance energy transfer, but not with external excitation.

Conventional quantum dots have the potential for significantly improved performance over organic fluorophores for *in vivo* imaging (reviewed in ref. 7). Their advantages include an exponentially increasing extinction coefficient towards the blue, potentially high quantum yield, precise tunability of emission wavelength, photostability, chemical stability and disease-specific targeting. At present, however, the widespread use of quantum dots in animal experiments and their eventual translation to the clinic for patient care are hindered by several fundamental problems.

First, all existing formulations of near-infrared quantum dots contain semiconductor or heavy metals with potential toxicity. Second, an organic coating is needed to render quantum dots soluble in aqueous environments. When the organic coating and its water of hydration are considered, the final hydrodynamic diameter of every quantum dot described to date is much larger than the renal filtration threshold. Hence, near-infrared quantum dots injected intravenously for cancer or other disease targeting cannot be cleared from the body. Indeed, secondary coatings, such as pegylation, often added to large quantum dots, merely delay the inevitable uptake by, and concentration in, the liver. Third, the potential advantage of increasing excitation towards the blue is actually lost in Rayleigh scattering tissue[8]. That is, the increasing scatter of tissue at bluer wavelengths offsets the increasing absorption of the quantum dot, resulting in net absorption similar to the single absorption peak of a conventional organic fluorophore.

Although the bioluminescent quantum dot described by So *et al.* solves the third fundamental problem, it still suffers from the first two, as well as several others. Imaging requires systemic administration of a foreign protein (luciferase) and a foreign enzyme substrate (coelenterazine), both of which have immunogenic potential and can alter the biological system under study. Light generation is completely dependent on the biodistribution of the enzyme substrate, and thus on the relative perfusion of the particular organ, tissue or tumor to be imaged. In addition, the large hydrodynamic diameter of the bioluminescent quantum dot will undoubtedly limit its ability to extravasate from the vasculature and thus target normal

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tissues or organs, and many tumors.

Nevertheless, bioluminescent quantum dot technology has the potential to greatly improve near-infrared fluorescence detection in living tissue. And it stimulates one to think about how the interconversion of energy from one form to another might solve major problems in the field of *in vivo* imaging.


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# Fancy footwork in the sequence space shuffle

Frances H Arnold

**Recent reports on directed evolution broaden the scope of evolutionary enzyme engineering.**

If we were able to explore all possible proteins, we would likely find fantastical molecules that might solve any number of human problems. The riches of sequence space include cures for cancer and solutions to the energy crisis, along with countless other valuable molecules. Unfortunately, such a comprehensive exploration is not even remotely possible, as sequence space is Vastly—for "Very-much-more-than-astronomically"—large[1]. Just a single copy of each 300-amino acid sequence, for example, would fill dozens of universes. That nature has explored only the tiniest fraction of this space over the entire history of life on Earth should leave protein engineers excited about their own opportunities for discovery.

Yet excitement over the untold riches of sequence space must be tempered by the recognition that the great majority of those sequences don't code for anything interesting; most don't even fold. Estimates for the density of functional proteins in sequence space range anywhere from 1 in $10^{12}$ to 1 in $10^{77}$. No matter how you slice it, proteins are rare. Useful ones are even more rare. This might lead one to believe that discovering new proteins by mutation and selection is highly unlikely and to discount evolution as an algorithm for discovery. So how do laboratory evolutionists discover new proteins on the timescale of a PhD thesis or, worse, a commercial deadline? They do it by taking the right kinds of steps and starting from the right places.

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Three recent reports describe new twists on this theme. Writing in the *Journal of the American Chemical Society*, Qian and Lutz[2] show how circular permutation might complement other, more tried-and-true steps in the search for better enzymes. In a report in *Nature Genetics*, Peisajovich *et al.*[3] provide a laboratory demonstration of how this permutation step might happen in nature. Finally, writing in *Science*, Park *et al.*[4] demonstrate how some 'rational design,' with inspiration from studying evolutionarily related proteins, can help find a good place to do the sequence space shuffle.

Evolution works because functional proteins are not evenly distributed in sequence space. Functional proteins are surrounded by other functional proteins that share the same overall structure. Even though most random amino acid substitutions are deleterious, many are not. Sometimes, a single substitution can improve a protein; accumulating such beneficial mutations over iterative rounds of mutagenesis and selection is an effective evolutionary strategy. Random mutation is only one search mechanism that explores sequence space efficiently. Recombination also accesses functional proteins with high probability and can make much larger jumps in sequence space than random mutation[5]. Laboratory evolutionists have also used less-natural search operations: saturation mutagenesis and random mutagenesis targeted to key portions of a protein (for example, the active site) are widely believed to provide advantages over more random approaches, especially when detailed structural information is available.

Qian and Lutz demonstrate that circularization and random opening should be included on a list of preferred search steps by making permutations on a lipase from *Candida antarctica* that is widely used in chemical synthesis. Postulating that the enzyme might hydrolyze bulkier substrates more efficiently if it had greater flexibility in its active site, these authors set out to determine whether opening up new C- and N-termini might provide that flexibility, especially if the new ends appeared near the active site. Working at the gene level, they connected the termini with a flexible linker, circularized the construct and proceeded to make random cuts. Screening for the genes that produced lipase activity in *Pichia pastoris* yielded functional permutants. Not only did they identify 63 new ways to start and stop the *C. antarctica* lipase, they also found some variants with significantly higher (10–60 fold) $k_{cat}$ values on lipase substrates (*p*-nitrophenol butyrate and 6,8-difluoro-4-methylumbelliferyl octanoate).

The net result of permutation is (presumably) a protein of the same overall structure, and with most of the amino acids in the same places in the structure. However, the sequences and topologies might be completely different if the polypeptide chain starts and ends at very different positions in the structure (**Fig. 1**). What role this strategy plays in the evolution of new functional proteins still remains to be determined, but it could wreak havoc for patent attorneys!

Various natural protein families bear the marks of having undergone permutation, leading to rearrangement of functional modules and diversification of their topologies. The circularization strategy used by Qian and Lutz to obtain their permutants is not likely to happen *in vivo*. However, Peisajovich *et al.* replicated what is considered the most likely natural equivalent of circular permutation—gene duplication and in-frame fusion followed by degradation from the 5′ and 3′ ends to generate new N- and C-termini. They tested this set of steps on a gene for a DNA methyltransferase (M.*Hae*III) and demonstrated that not only could this mechanism produce active permuted methyltransferases, it could do so through a series of functional intermediates in which only the N- or the C-terminus was degraded. These intermediates, which contained some wholly or partially duplicated modules, folded and functioned, albeit at a reduced level compared with the unmodified protein. Because all of the steps used for this laboratory demonstration have natural counterparts, it is likely that similar events can and

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