it has been unclear whether human immunoglobulins alone could support bovine humoral immunity in the absence of endogenous bovine immunoglobulins.

These formidable challenges make the achievement of a transgenic calf expressing high levels of human antibodies all the more remarkable. Kuroiwa *et al.*[5] focused first on successively inactivating all the bovine IgM heavy chain genes (**Fig. 1**), whose expression is essential for B-cell development. Because ruminants, unlike mice and humans, have two functional IgM loci (*IGHM* and *IGHML1*), four alleles had to be targeted to knock out IgM heavy-chain production. In the next step, the $IGHM^{-/-}$ $IGHML1^{-/-}$ fibroblasts were transfected with an artificial human chromosome (κHAC) bearing the unrearranged human heavy and κ-light-chain loci (**Fig. 1**). In the end, after seven rounds of cloning, a healthy transgenic calf, with all bovine IgM heavy chain alleles inactivated and bearing the human artificial chromosome, was obtained. This animal expressed 60-fold more human immunoglobulins than animals described previously[8]—a yield that is potentially competitive from a cost perspective with producing nonhuman hyperimmune globulins.

Vaccination of the calf with anthrax-protective antigen yielded high titers of anthrax-specific immunoglobulins. Although ~80% of serum IgGs were functional chimeric antibodies comprising human heavy chains and bovine light chains, the remainder were fully human (**Fig. 1**). Once purified, the hyperimmune globulins fully protected mice challenged with anthrax spores and in an *in vitro* toxin neutralization assay outperformed a control anthrax hyperimmune globulin preparation derived from human donors.

Importantly, the calf's immunization response was similar to that of wild-type cattle, confirming that the human immunoglobulin loci can support the humoral response in the absence of bovine IgM. Other transgenic calves produced in this study appeared to produce similar levels of human IgGs, suggesting that a herd of cattle with this genotype could provide an abundant source of human hyperimmune globulins. Nevertheless, extensive purification will be necessary to obtain preparations containing only fully human IgGs, which will increase production costs. Knocking out the bovine Igλ locus, which contributes ~90% of light chains in cattle, in this line could further increase the proportion of fully human immunoglobulins and improve process yields.

Although the seven years that have elapsed since this group reported transchromosomic calves expressing human immunoglobulin loci[8] might seem like a long development time, it is worth remembering that the line generated in the present study[5] or subsequent lines could support production of multiple products. Each new hyperimmune globulin product would be dependent on the antigen used in the immunization protocol, rather than the bovine line or the purification process. Scale-up should be relatively straightforward, although this might require cloning rather than natural breeding.

It is still too early to confidently predict the commercial success of human hyperimmune globulins from transgenic cattle. Uncertainties remain concerning, for example, the impact of purification on production costs and the feasibility of using somatic cell nuclear transfer to generate large numbers of animals. Clinical studies—the costliest and riskiest aspect of drug development—must also be completed. But given the flexibility and scalability of using transgenic large animals, this approach may be well placed to compete with traditional human- and animal-derived intravenous immunoglobulins, hyperimmune globulins, and monoclonal and polyclonal antibodies produced in cell culture[9] in applications spanning infectious diseases, oncology, neurological conditions and immune modulation. As we enter the Chinese year of the ox, it seems fitting to look forward to clinical trials of polyclonal antibodies obtained from transgenic cattle.

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One photon up, one photon down

Enrico Gratton & Michelle Digman

A microscopy technique based on stimulated Raman scattering achieves label-free imaging with very high sensitivity.

Identifying different molecular species in microscopic images is still a considerable challenge in many areas of biology. Most commonly, especially in experiments on live cells and tissues, what is detected is not the native molecule but a fluorescently labeled analog, which is assumed to mimic the behavior of the unlabeled molecule. In a recent *Science* paper, Xie and colleagues[1], describe a new techniqúe, stimulated Raman scattering (SRS) microscopy, that is capable of imaging unlabeled molecules in live cells and tissues with diffraction-limited resolution and high sensitivity.

Since the 1920s, when Chandrasekhara Raman[2] first explained the loss of energy to vibrations from a beam of monochromatic light traversing a liquid sample, investigation of the quasi-elastic interactions of light with matter has become a major technique for analyzing the vibrational spectrum of molecules in the condensed state and the composition of biological samples[3]. The advent of the laser in the 1960s and the introduction of resonance Raman scattering, which increase the sensitivity to specific vibrations near a chromophore, made this a relatively simple and accessible method. The appeal of this approach in microscopy is that the molecular vibrations excited by the Raman effect are exquisitely dependent on local molecular arrangements and therefore serve as a fingerprint of individual molecules or classes of molecules.

Until now, the small intensity of Raman scattering has meant that only techniques based on coherent anti-Stokes Raman scattering (CARS) have had the sensitivity necessary for diffraction-limited microscopy[4]. In the CARS effect, two laser beams impinge on the sample. The pump laser excites a vibration and the probe laser produces the anti-Stokes transition (that is, addition to the probe laser of energy contained in excited molecular vibrations), resulting in a new emission wavelength different from those of the pump and probe lasers. This new wavelength characterizes the energy of the

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vibration that was added to the probe beam, and it can be detected with a very high signal-to-noise ratio. During the 1990s, Xie's group combined CARS with laser scanning microscopy to achieve diffraction-limited resolution and high sensitivity by exposing a sample to collinear laser beams of different energy[4]. However, in CARS microscopy, attribution of the signals to specific vibrations and therefore to specific molecules remains difficult owing to signal distortion and the relatively large nonresonant background.

In their new work, Xie and colleagues[1] show that these problems can be overcome using SRS microscopy. In an SRS experiment, two laser beams that differ by exactly the energy needed to excite a specific molecular vibration impinge simultaneously and collinearly on the sample (**Fig. 1**). One laser is used to pump the vibrations and the other to produce the stimulated emission process that forces these vibrations to return to their ground state. The authors demonstrate that the spectral response of SRS is identical to the spontaneous Raman signal and that the background signal is eliminated, making it possible to use the extensive Raman literature to assign vibrational spectra to specific molecules.

Xie and colleagues[1] achieve very high sensitivity, which is crucial for imaging biological samples, by a clever detection set-up based on modulation of the pump and stimulated laser beams. In their design, the laser used for stimulated emission is modulated at high frequency, and this modulation transfers to the pump beam if molecules with the particular Raman excitation band are present. This transfer of modulation from one beam to another is detected using a lock-in amplifier, delivering very high sensitivity. Because two laser beams are interacting in the sample, all the advantages of multiphoton microscopy, such as optical sectioning and diffraction-limited resolution, can be obtained without using pinholes[5]. The pump and probe beam are then scanned through the sample using conventional galvanometer scanners, and the changes in the modulation of the pump beam are recorded at the different positions in the sample. The amplitude of the modulation at each pixel is proportional to the concentration of the molecules with the particular Raman absorption band. Tuning to a specific vibration is done by changing the difference in energy between the pump and probe laser beams.

The idea of applying different laser beams of different energy to produce specific interactions in the sample is not new and has been used with direct two-photon excitation of electronic states[5], the CARS effect[5] and stimulated emission[6]. All these schemes allow optical sectioning without a pinhole and very high selectivity and sensitivity. One important difference between two-photon excitation and the stimulated emission methods (either from vibrational or from electronic states) is that the stimulated emission methods depend separately and linearly on the intensity of the pump and probe beams. Therefore, only one of the two laser beams has to be of relatively high power, reducing instrument cost and sample damage. From the technical point of view, we anticipate that other techniques for modulating the laser beams, such as those used by Dong *et al.*[6] for their stimulated emission microscope, will further improve the signal-to-noise ratio. One technical limitation of SRS microscopy as proposed by Xie and colleagues[1] is that the pump and probe beams are measured in the same direction as that of the laser propagation. This can be problematic in the case of thick tissues, although improved detection systems could alleviate this limitation.

Given its high selectivity for specific vibrations, SRS microscopy lends itself in principle to the study of a large variety of molecules, including metabolites and small-molecule drugs. A potential limitation of the method is that the low intensity of Raman scattering requires relatively high concentrations of the molecule of interest. Xie and colleagues[1] estimate that the detection limit for their model substrate retinol is 50 μM, although this value will vary widely according to the scattering cross-section of the particular molecule of interest.

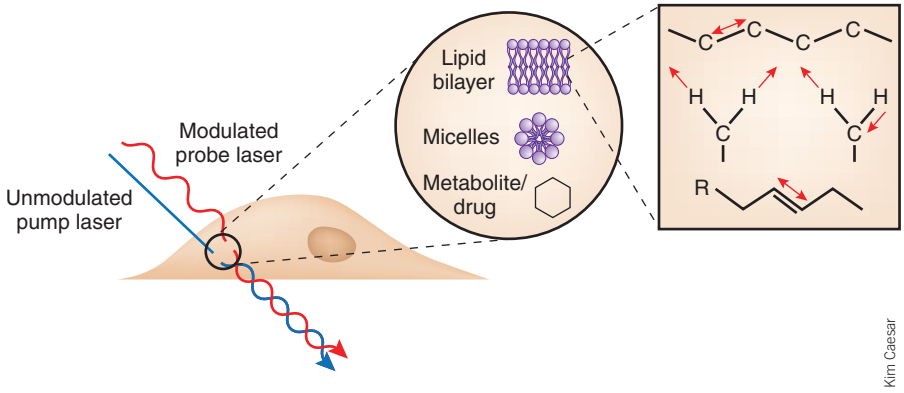

Figure 1 Before passing through the sample, the pump laser (blue) is unmodulated and the probe laser (red) is modulated, that is, its amplitude is varied with a high frequency. The lasers are focused on different areas of the sample with different molecular compositions. When an area contains molecules with vibrations of the energy equivalent to the energy difference between the two lasers, the modulation is transferred from the probe to the pump laser. The amplitude of the modulation, which is proportional to the concentration of the molecule of interest, can be measured with high sensitivity.

The concentration of vibrations that can be measured in a reasonable amount of time by SRS is adequate to detect vibrations arising from lipids, as these molecules are very abundant in biological samples. The sensitivity of SRS to specific lipids is of great interest as the detection and characterization of lipids in biological tissues using fluorescent molecules is still challenging. Fluorescence-based techniques rely on the assumption that fluorescent lipid analogs behave exactly as their nonfluorescent original molecules, which is difficult to demonstrate. By circumventing this issue, the SRS technique may contribute substantially to our knowledge of the microscopic organization and local composition of biological membranes. In addition, SRS will be useful for studying lipid metabolism and transport pathways, either by monitoring the appearance and disappearance of characteristic vibrations at different subcellular locations or by characterizing the composition of transport intermediates.

A promising application of SRS is the study of drug delivery to complex tissues. In a proof-of-principle experiment, Xie and colleagues[1] compare the transport characteristics of retinoic acid and dimethyl sulfoxide in mouse skin. This suggests that Raman microscopy has the unique ability to visualize the penetration and sites of accumulation of unlabeled compounds *in situ*.

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