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In vivo imaging of green fluorescent protein-expressing cells in transgenic animals using fibred confocal fluorescence microscopy

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Abstract

Animal imaging requires the use of reliable long-term fluorescence methods and technology. The application of confocal imaging to in vivo monitoring of transgene expression within internal organs and tissues has been limited by the accessibility to these sites. We aimed to test the feasibility of fibred confocal fluorescence microscopy (FCFM) to image in situ green fluorescent protein (GFP) in cells of living animals. We used transgenic rabbits expressing the enhanced GFP (eGFP) gene. Detailed tissue architecture and cell morphology were visualised and identified in situ by FCFM. Imaging of vasculature by using FCFM revealed a single blood vessel or vasculature network. We also used non-transgenic female rabbits mated with transgenic males to visualise eGFP expression in extra-foetal membranes and the placenta. Expression of the eGFP gene was confirmed by FCFM. This new imaging technology offers specific characteristics: a way to gain access to organs and tissues in vivo, sensitive detection of fluorescent signals, and cellular observations with rapid acquisition at near real time. It allows an accurate visualisation of tissue anatomical structure and cell morphology. FCFM is a promising technology to study biological processes in the natural physiological environment of living animals.

Keywords: In vivo imaging; Green fluorescent protein; Fibred confocal microscopy; Transgenic rabbit

Introduction

Advances in the understanding of animal development and physiology require the use of reliable long-term fluorescence in vivo imaging methods and technology. In recent years, the techniques of fluorescence, which use fluorophores and their excitation by laser, have contributed to the development of small animal imaging in vivo. Confocal microscopes that use miniaturised optic fibres to deliver light and to measure either reflected or excited fluorescence light were used to image biological tissues in situ and in vivo (Gmitro and Aziz, 1993; Delaney et al., 1993; Juskaitis et al., 1997; Ilyin et al., 2001; Helmchen et al., 2001; Sung et al., 2002). However, optic confocal imaging of organs and tissues has been limited by the difficulty of gaining access to these sites in living animals (Helmchen, 2002). Recently, fibred confocal fluorescence microscopy (FCFM) has been conceived for in vivo and in situ imaging thanks to optical mini-probes. This new imaging technology offers the possibility of detecting in situ fluorescent signals and has been used to visualise

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blood circulating cells and the microvasculature (Laemmel et al., 2004), epithelial cell types (D'Hallewin et al., 2005) and nuclear DNA fragmentation in cells undergoing apoptosis (Al-Gubory, 2005) in living animals.

The other main barrier for in vivo imaging is that fluorescent dyes must be used to yield stable and sufficient signal without causing any damage to biological tissues. Fibre optic confocal imaging technologies have been used in rodent animal models to visualise: blood vessels and nerves in the rat vas deferens and colon (Papworth et al., 1998), subsurface keratinocytes, blood vessels and nerves in hairless mouse skin (Bussau et al., 1998), subsurface human melanomas implanted in athymic mouse (Anikijenko et al., 2001), early subsurface changes in the mucosal architecture of the colon in a rat model of ulcerative colitis (McLaren et al., 2001), and different epithelial cell types in rat bladder epithelium (D'Hallewin et al., 2005). These investigations were based on the application of exogenous fluorescent dyes. However, removal of dyes by the vascular and the lymphatic networks, enzymatic degradation of dyes and permeability of living cells, as well as the toxicity of the dyes constitute so many limiting factors that affect staining in vivo and do not allow reliable and continuous fluorescence imaging in vivo.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become the most widely used reporter protein in living organisms (Prasher, 1995). The advantages of GFP are that its fluorescence is stable and species independent, and does not need exogenous substrates or cofactors. These properties have made GFP a powerful imaging marker to study gene expression, protein localisation, receptor internalisation and recycling, and cellular secretory events (Chalfie et al., 1994; Kain et al., 1995; Kishimoto et al., 2000; Yamazaki et al., 2000; Conway et al., 2001; Zhang et al., 2002).

In the present study, the feasibility of FCFM to image in vivo and in situ cells expressing GFP in organs and tissues of transgenic rabbits was investigated. We also used non-transgenic female rabbits mated with transgenic males expressing GFP in order to visualise in vivo and in situ GFP-expressing cells in the foetal membranes and the placenta.

Material and methods

Experimental animal model

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorisation no. 78–34). We choose a mutant form of the GFP called enhanced GFP (eGFP), which fluoresces 35-fold more intensely than wild type (wt) GFP when excited at 488 nm (Cormack et al., 1996). The study was carried out on two transgenic rabbits expressing the eGFP gene and on three non-transgenic female rabbits mated with transgenic males expressing eGFP. The transgenic animals were obtained by the technique of pronuclear microinjection of DNA (Hammer et al., 1985) into naturally ovulated and fertilised rabbit eggs. The DNA construct contained the promoter of the ubiquitously expressed EF1 alpha gene, the eGFP gene, the PAC gene coding for a puromycin resistance protein under the control of an internal ribosome entry site (Taboit-Dameron et al., 1999) and the insulator sequence 5′HS4 added before the promoter (Giraldo et al., 2003). From each transgenic founder rabbit, F1 was obtained and used for in vivo imaging of eGFP-expressing cells. Transgenic F1 newborns were identified by the visualisation of eGFP fluorescence in eyes using a simple blue-light-emitting diode flashlight and a bypass emission filter.

Fibred confocal fluorescence microscopy (FCFM)

Fibred confocal fluorescence microscopy (FCFM), also known as Cell-viZio (Mauna Kea Technologies, Paris, France), was recently conceived and developed for in situ and in vivo imaging. FCFM instrumentation and processing have been recently described in detail (Laemmel et al., 2004). Briefly, the FCFM is built up on an optical principle of confocal microscopy, which is the ability to reject light from out-of-focus planes and to provide a clear in-focus image of a thin section within the sample. FCFM is based on the excitation of either intrinsic or extrinsic fluorescent molecules in the examined tissue. FCFM is composed of a laser scanning unit, LSU-488 (FibroScan), a range of optical fibre mini-probes (ProFlex), which are the link between the scanning device and the micro-objective, and a software unit to control the system and to manage the image data (ImageCell). FibroScan uses a laser source with a wavelength of 488 nm which is scanned by two mirrors on the proximal surface of a fibre bundle. It is possible to achieve near-real-time imaging (12 frames per second) using a 4-kHz oscillating mirror for horizontal line scanning and a galvanometric mirror for frame scanning. Sequentially injected laser in each fibre is focused on the tissue via a distal micro-objective of the probe. The fluorescence collected by the micro-objective is then re-injected in the same fibre used for illumination. ProFlex is a flexible mini-probe composed of tens of thousands of optical fibres. The confocality of the system derives from the size of each fibre core. Indeed, a 2-μm core diameter serves as a pinhole for both excitation and collection, giving the probe its optical slicing capability. The available optical probes have a