

RESEARCH ARTICLE

In Vivo Fluorescence Imaging is Well-Suited for the Monitoring of Adenovirus Directed Transgene Expression in Living Organisms

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Abstract

Purpose: We tested a new light detection cooled charge-coupled device (CCD) for *in vivo* assessment of noninvasive, whole-body fluorescence optical imaging of adenovirus directed enhanced green fluorescent protein (AdEGFP) expression.

Procedures: AdEGFP was injected i.v. into BALB/c mice via tail vein. Whole-body fluorescence optical imaging of AdEGFP expression was performed using a Kodak 2000MM Image Station before and after vector administration.

Results: EGFP expression was exclusively detected around the abdominal cavity, and the fluorescent signal peaked at day 4 and then remained detectable for at least 30 days. *Ex vivo* fluorescence imaging confirmed that EGFP expression was restricted to the liver, and transgene expression was homogeneously diffused into all four lobes.

Conclusions: These findings demonstrate that *in vivo* fluorescence imaging provides functional data indicating the approximate location, magnitude, and duration of AdEGFP expression.

Key words: *In vivo* imaging, CCD camera, EGFP, Adenoviral vector, Gene therapy

Introduction

Noninvasive molecular imaging is an attractive tool for the external detection of transgene expression in living animals. For gene therapy studies, *in vivo* monitoring and quantification of transgene expression is an important approach to determine the intensity of therapeutic gene expression in targeted tissues in disease models. The technique provides an opportunity to evaluate new gene

therapy strategies for the treatment of human diseases. Detecting the location, magnitude, and time variation of reporter gene expression without using invasive techniques, like immunohistological staining or enzyme activity assays, greatly facilitates the follow-up of biological processes in living organisms [1]. In addition, the number of animals required per experiment can be reduced using live animal *in vivo* imaging methods. Moreover, following transgene expression in the same animal over time can decrease the variation depending on the organism being used and increase the power of statistical analysis [2].

Fluorescence optical imaging is a very promising tool based on external light emission from a light source and

excitation of the fluorophore in the transfected tissue. The emitted fluorescent light is subsequently detected by specialized cooled charge-coupled device (CCD) cameras that quantify the fluorescent intensity and represent the signal in the image [3]. The most commonly used fluorescent proteins are green fluorescent protein (GFP) and the red fluorescent protein. Unlike luciferase, fluorescent proteins do not require any substrate to be visualized [4]. In addition, luciferin is very expensive and has a short half-life requiring immediate visualization within half an hour of administration. Since luciferases are oxygenases, the oxygen need within the lumen of the gut or necrotic cores of large tumors and hypoxia may limit the use of luciferases as reporters in anaerobic environments [5]. Absorption and scattering of light by mammalian tissues can easily complicate bioluminescent imaging as well [6]. By comparison, GFP is non-toxic and does not interfere with normal cellular activity. When the reporter gene is expressed, fluorescence imaging enables visualization of the dynamic processes inside cells, permitting longitudinal monitoring of a disease process in living organisms [7]. Optical imaging has significant advantages when compared to other *in vivo* imaging modalities such as positron emission tomography or MRI [8]. For example, optical imaging agents are of low cost and does not require radioactive substrates or contrast agents for visualization.

Previous reports have shown that fluorescence optical imaging technology has been used to demonstrate the transgene expression [9], microbial infection [10], and tumor growth/metastasis [11] in experimental animals. In the present study, we applied fluorescence optical imaging for an *in vivo* assessment of adenovirus-mediated transgene expression in BALB/c mice. For this purpose, a first generation recombinant adenoviral vector encoding the enhanced GFP cDNA (AdEGFP) was administered through the tail vein. After systemic virus delivery, transgene expression was periodically followed by CCD camera.

Materials and Methods

Animals

Ten- to 12-week-old male BALB/c mice, weighing 25–30 g, were obtained from the Laboratory Animal Care Unit of Akdeniz University Hospitals and Clinics. Animal experiments were conducted in accordance with the Institutional Animal Care Guidelines.

Preparation of Adenoviral Vectors

E1/E3-deleted first generation recombinant adenoviral vector-expressing EGFP cDNA, under the control of a cytomegalovirus promoter (AdEGFP), were propagated in HEK293 cells and purified by CsCl banding [12]. The titers of adenoviral stocks

were determined as 10^{13} DNA particles per milliliter by spectrophotometric measurements (A_{260} readings). Adenoviral vectors were stored at -80°C in 10 mM Tris-Cl containing 20% glycerol. Viral stocks were desalted through a PBS-Sephadex G-50 (Sigma-Aldrich, St. Louis, MO) column to remove glycerol prior to injections [13]. The functional titers of adenoviral stocks were determined by plaque titrating on 293 cells and expression assays for encoded proteins. Typically, the particle/plaque forming unit ratio was 25.

Systemic Vector Administration into BALB/c Mice

BALB/c mice received intraperitoneal (i.p.) anesthetics, a mixture of ketamine/xylazine (100:10 mg/kg). Various doses of AdEGFP (10^{10} , 3×10^{10} , 10^{11} , and 2×10^{11} DNA particles/mouse) in sterile PBS (final volume of 200 μl) were administered i.v. via lateral tail vein using a 26-g syringe. Control animals were injected with 200 μl PBS only.

In Vivo Fluorescence Imaging

After AdEGFP administration, mice were imaged at various time points using a cooled CCD camera (Kodak 2000MM Image Station; Eastman Kodak Company, New Haven, USA). To acquire the images, mice were anesthetized, and the abdominal regions to be imaged were shaved. Next, anesthetized mice were placed in a light tight chamber and grayscale photographs were taken using 0.05 s exposure time using open filters. Keeping mice at the same position, fluorescence images were also collected using 60 s exposure time with 465 excitation/535 emission filters but no binning. Focus, field of view, and aperture (*f*-stop) settings of the lens and exposure time were kept identical for the comparison purposes. For confirmation of the *in vivo* imaging, mice were killed 4 days after 2×10^{11} AdEGFP particle administration. Major organs, especially liver, were analyzed *ex vivo* by CCD camera. To test the scenario of tissue obstruction, the abdominal skin and peritoneal wall were surgically removed 1 day after 2×10^{11} AdEGFP particle injection, and images were taken after every step.

Quantification of Fluorescence Signals

Images were analyzed using Kodak 1D Software 3.6.5 (Eastman Kodak Company, New Haven, USA). Regions of interest (ROIs) were defined using a standard area in each image. Fluorescence signal intensity was recorded as a net intensity reflecting the sum of background subtracted pixels within the ROI. Average net intensity was calculated by repositioning the mouse and taking at least three measurements. When liver lobes were analyzed *ex vivo*, mean intensity values for each lobe were used to obtain the average intensity of the signal region. Fluorescence grayscale images were artificially colored for depiction purposes. Pseudocolor depicts the light intensity (red and purple indicate maximum and minimum light intensity, respectively). These fluorescence pseudocolored images were superimposed on the grayscale photographs for anatomical location. All assays were repeated twice to confirm the observations.

Results

Non-invasive Fluorescence Optical Imaging of BALB/c Mice Generated Strong Signals Only Around Abdominal Cavity

To obtain measurable levels of fluorescent intensity; various doses (10^{10} , 3×10^{10} , 10^{11} , and 2×10^{11} particles) of AdEGFP were injected into BALB/c mice i.v. via tail vein. Imaging was subsequently performed periodically using CCD camera. Representative images from mice receiving 2×10^{11} particles

demonstrate that the maximum intensity of transgene expression was observed 4 days after injection (Fig. 1a), gradually decreased over the next 17 days, and was barely detectable on day 30 (Fig. 1b). In addition, the fluorescent signal in these mice emanated exclusively from the upper abdominal cavity. Only closer monitoring (FOV 30) at day 21 could detect the weak signal around the abdomen. Analysis of the fluorescent signal at the different doses on day 4 clearly indicates a dose-dependent increase in fluorescence intensity (Figs. 2a, b). Furthermore, this analysis also determined that delivery of

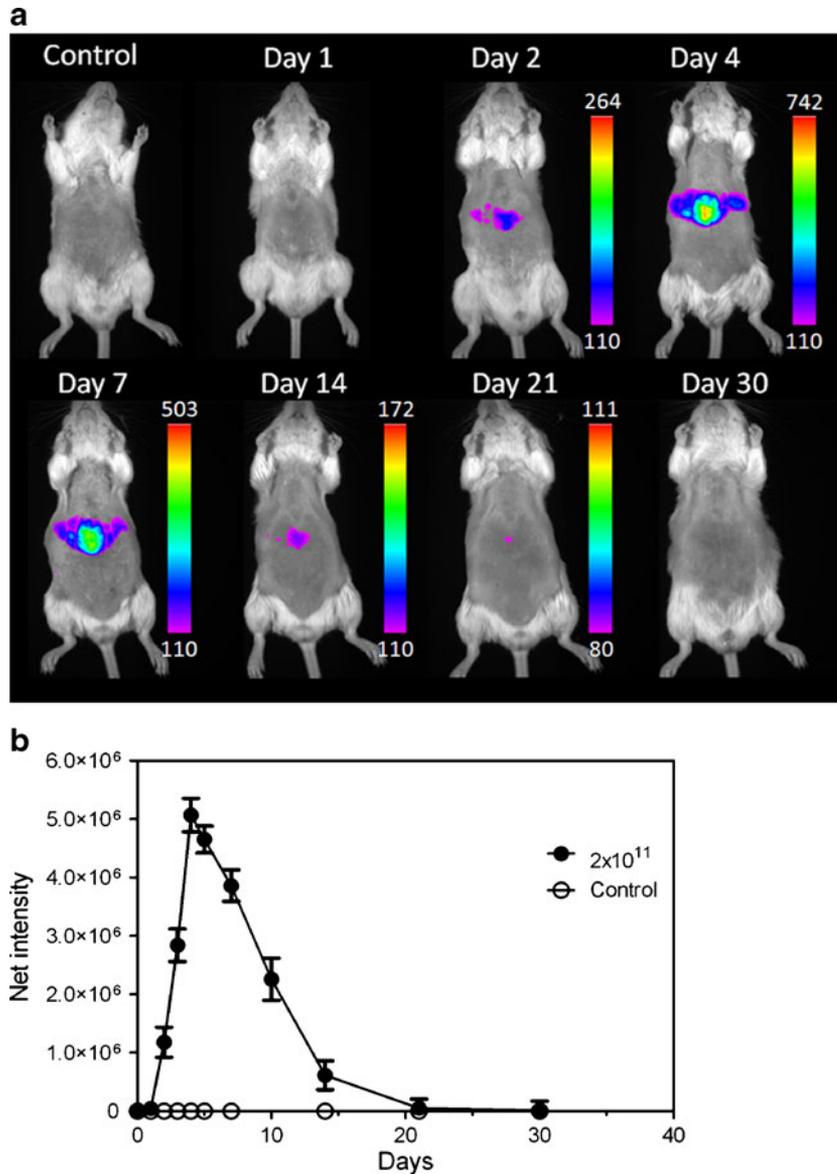


Fig. 1. *In vivo* fluorescence imaging. **a** Time course of transgene expression in mice after systemic AdEGFP vector delivery. Representative composite images of CCD recorded views of a mouse injected with the maximum dose of AdEGFP vector (2×10^{11}) displaying the tissue distribution pattern of EGFP expression over time. Whole-body CCD images were acquired at various time intervals (days 1, 2, 4, 7, 14, 21, and 30) after injection of 2×10^{11} virus particles. **b.** Quantitative analysis of the fluorescence intensity after 2×10^{11} AdEGFP injection. The data relating to lower doses of AdEGFP (1×10^{10} , 3×10^{10} , and 1×10^{11} virus particles/mouse) injections were omitted from the graph for clarity purposes. Error bars represent \pm SEM of five animals/dose.

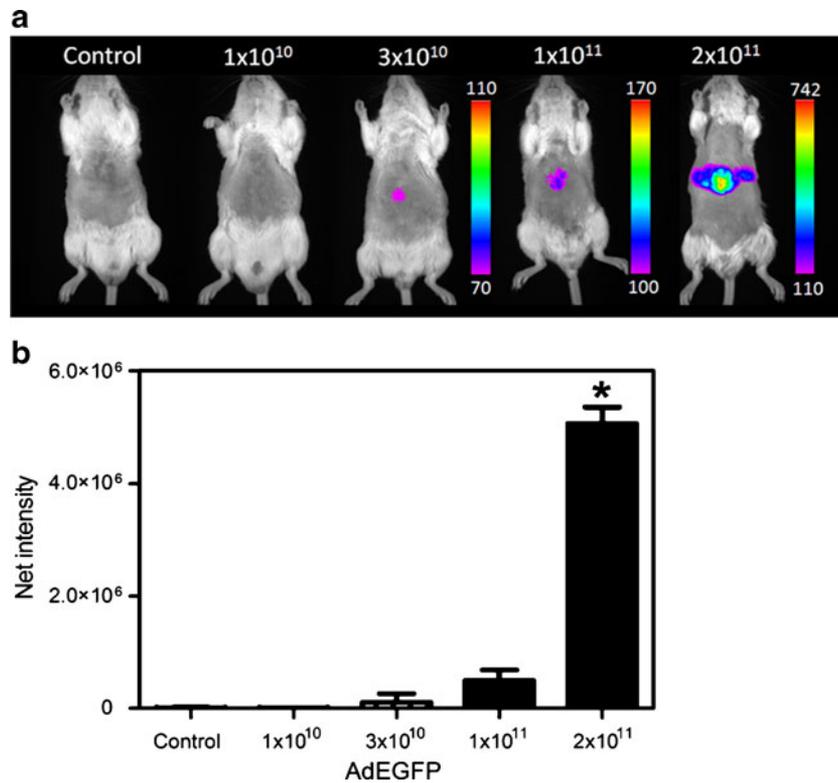


Fig. 2. a Whole-body CCD images of mice showing EGFP transgene expression 4 days post-injection. Pseudocolor images of fluorescent signals were overlaid on grayscale photographs recorded using Kodak 2000MM Image Station. The titers of AdEGFP vector injected into mice are shown *above the pictures* and the *color bars* display relative intensity. The maximum and the minimum intensity values are provided on the *top* and on the *bottom* of the color bars. **b** Quantitative measurement of AdEGFP transgene expression using CCD camera. *Error bars* represent \pm SEM data from five mice. $*p < 0.05$.

3×10^{10} AdEGFP particles per mouse was the minimum amount needed to attain reproducibly detectable transgene expression. Mice receiving 10^{10} AdEGFP particles or PBS yielded no detectable fluorescent signal by this approach. Quantitative analyses of the fluorescent signal intensity are

shown in Fig. 2b. One-way ANOVA followed by Bonferroni's multiple comparison test indicated that only the delivery of 2×10^{11} AdEGFP DNA particles yielded a statistically significant fluorescent signal compared to mice injected either with PBS or lower doses of AdEGFP vector.

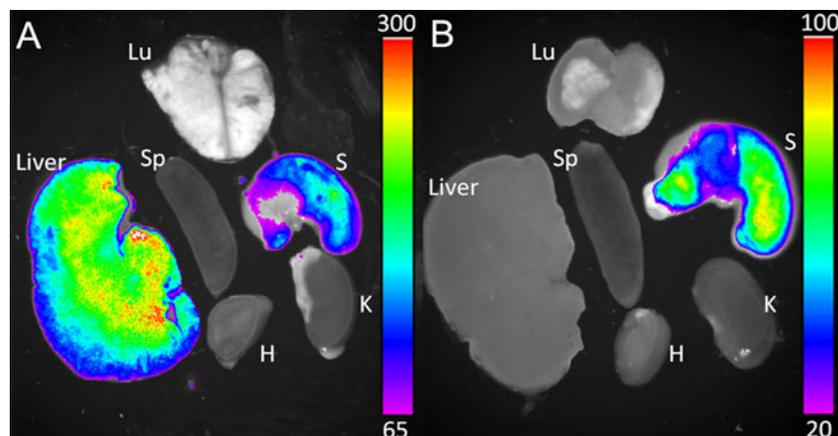


Fig. 3. Ex vivo imaging of major mouse organs using CCD camera. Either 2×10^{11} virus particle (a) or PBS as a control (b) was systemically delivered into BALB/c mice through tail vein. Major mice organs (liver, left lateral lobe, Sp spleen, Lu lung, S stomach, H heart, K kidney) from six independent animals were then removed and the organ distribution of transgene expression was measured 4 days after injection.

Ex Vivo Localization and Analysis of Fluorescent Signals Following Vector Administration

To localize the fluorescent signal coming from the abdominal cavity following vector administration, mice were killed 4 days after injection of 2×10^{11} AdEGFP particles, and their major organs (liver, spleen, lungs, stomach, heart, and kidneys) were removed. Whereas fluorescent signals were detected from the stomach of mice injected with either AdEGFP or PBS (and thus considered to be autofluorescence), only the liver produced a fluorescent signal in the AdEGFP-injected mice (Figs. 3a, b). No fluorescent signal was detected from heart, spleen, kidneys, and other organs analyzed (data not shown).

Tissue Distribution Profile of Transgene Expression in Liver

To document the distribution pattern of EGFP transgene expression in the liver, BALB/c mice were killed 4 days after

systemic administration of 2×10^{11} particles/mouse of AdEGFP. Liver lobes of these mice were dissected individually, and fluorescent measurements were recorded from the each lobe *ex vivo*. A homogenous, but diffuse, EGFP expression profile was detected in each lobe (Fig. 4a). These results were also confirmed by quantitative measurements of mean fluorescent intensity recorded from the each liver lobe (Fig. 4b). One-way ANOVA analysis, followed by Bonferroni's multiple comparison test, indicates that the mean intensity lobe-specific fluorescent signals were not statistically different from each other ($p > 0.05$).

Tissue Obstruction Appears to be a Major Barrier in Non-invasive Fluorescent Imaging Using CCD Camera

To determine the degree of signal obstruction caused by tissues, BALB/c mice were injected with 2×10^{11} AdEGFP particles, and CCD recordings were performed 24 h after injection—a time when minimal transgene expression was detected (Fig. 1). Anesthetized mice were shaved, and tissue

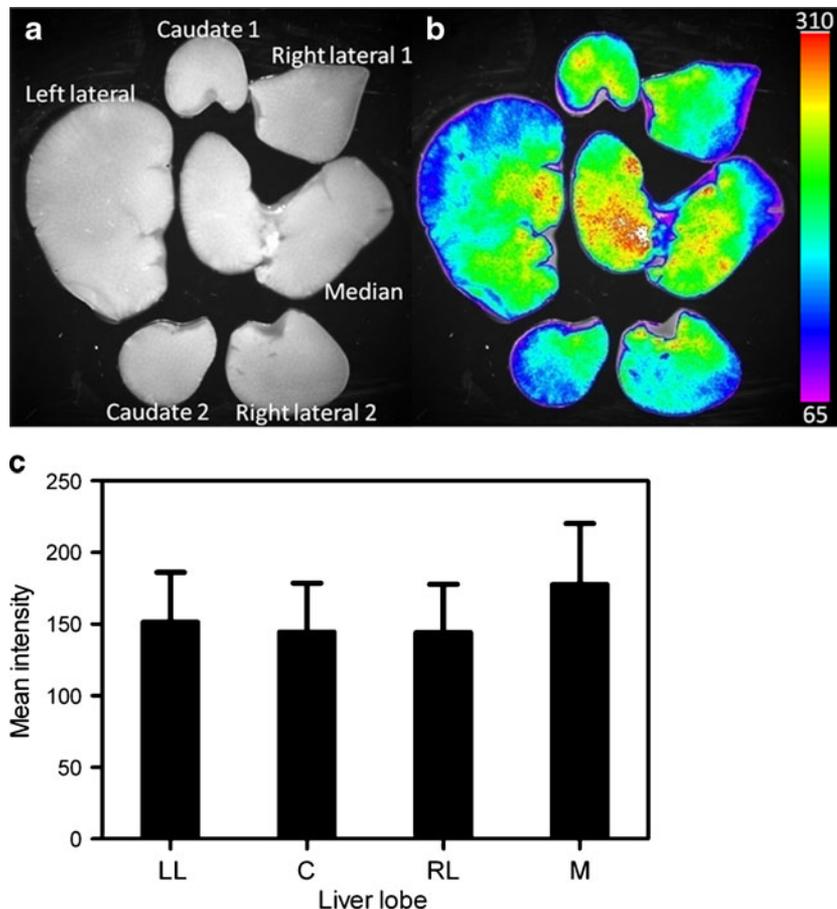


Fig. 4. Quantification of transgene expression among liver lobes. Four days after the systemic injection of 2×10^{11} AdEGFP particles, mouse livers were excised, and the liver lobes were separated for quantification purposes. Bright field images of liver lobes were photographed (a), and then fluorescence and grayscale images were overlaid on top of each other (b). Right lateral lobes and caudate lobes of liver were divided into two portions for the proper measurement of intensity using CCD camera. **c** The mean \pm SE fluorescence signal intensity recorded from four liver lobes for $n=6$ different animals, namely left lateral (LL), caudate (C), right lateral (RL), and median lobes (M). Error bars represent \pm SEM data from four mice.

layers were removed progressively. After each step (shaving, skin removal, and peritoneal membrane removal), mice were imaged. There was a 19% increase in net fluorescent intensity with skin removal, and a 36% increase of net fluorescent intensity with peritoneal membrane removal on average (Fig. 5). Thus, there was a significant difference among the groups that were just shaved, skin removed, and peritoneal membrane removed. Conversely, control animals showed no transgene-related signal, while only a weak autofluorescence signal was detected in the intestine, gall bladder, and urinary bladder. One-way ANOVA analysis followed by Bonferroni's multiple comparison test indicated that, although no statistically significant difference existed between skin-removed animals (panel B) versus peritoneal wall-removed animals (panel C), net fluorescent signal intensity of these two groups were statistically higher than rest of the groups tested. There was no statistical difference in net fluorescent intensity between shaved animals and peritoneal wall-removed control animals.

Discussion

Adenoviral vectors have many properties that make them suitable for use in gene therapy [14]. For example,

adenoviruses can infect a broad range of quiescent and proliferating cells with high efficiency [15]. In addition, robust transgene expression is achievable using adenovirus vectors following transduction [16]. These characteristics, together with their relative ease of preparation and high cloning capacity, have led to their extensive use as gene therapy vectors [17]. Currently, adenoviral vectors are the most extensively used vectors in clinical gene therapy trials worldwide [18, 19]. Gene therapy studies require information about the level and the duration of transgene expression, which are essential parameters to obtain a therapeutic benefit. Because of this reason, the use of noninvasive molecular imaging techniques has become very popular. Fluorescence optical imaging has some advantages over bioluminescence imaging, such that it does not need a substrate or a cofactor for visualization [20, 21]. In this technique, the fluorescence intensity of the transfected organs can be efficiently detected by CCD camera. In addition, images are gathered from superficial tissue sites by employing very short integration times [22]. Collectively, the technique of fluorescence optical imaging is a simple, inexpensive, and applicable noninvasive method for the detection of transgene expression in live animals. For this reason, we studied a non-invasive method to monitor

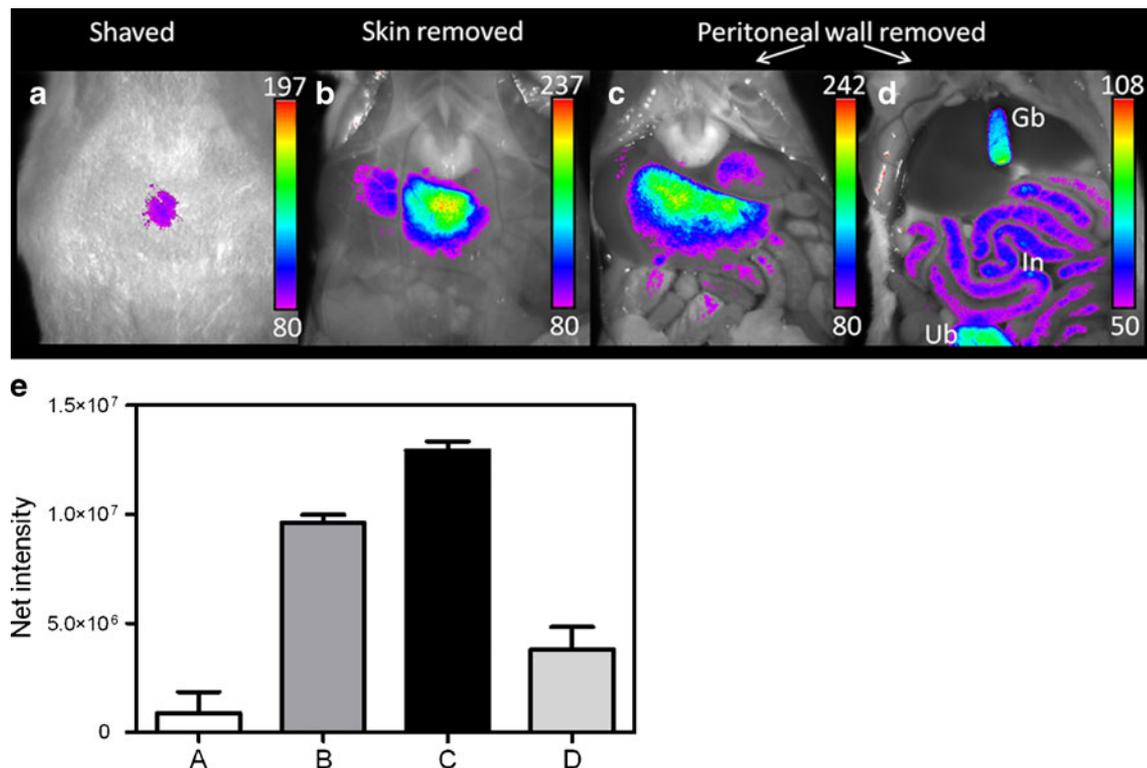


Fig. 5. Tissue obstruction of fluorescent signal detected by CCD camera. Mice were killed 24 h after injection of 2×10^{11} AdEGFP particles systemically via the tail vein. First, abdominal skin was shaved, followed by skin removal, followed by surgical removal of the peritoneal membrane. After every step, mice were imaged as **a** shaved, **b** skin removed, **c** peritoneal membrane removed, and **d** control mouse with peritoneal membrane removed. Control animals displayed autofluorescence signal only in intestine (*In*), gall bladder (*Gb*), and urinary bladder (*Ub*). **e** Quantitative measurement of fluorescent signals. Each bar represents \pm SEM of four mice. The *graph bars* show groups that were shaved (*column A*), skin removed (*column B*), peritoneal membrane removed (*column C*), and the control group (*column D*).

fluorescent gene expression directed by adenovirus vectors using EGFP as a fluorescent reporter in BALB/c mice.

For this purpose, an EGFP-encoding adenoviral vector (AdEGFP) was injected at increasing doses into BALB/c mice, and only the administration of 2×10^{11} DNA particles of AdEGFP generated a strong fluorescent signal detectable by a CCD camera. External transgene-related signal coming only from the upper abdominal cavity was maximal 4 days after vector administration, and then the signal gradually faded by day 21. The magnitude of the fluorescence intensity, a reflection of transgene expression, was critically dependent on the viral dose administered. As shown in two other previous studies, 2×10^{11} DNA particles of AdEGFP delivered through tail vein efficiently transduced liver cells as demonstrated by *ex vivo* fluorescent microscopy of liver sections [23, 24]. Successful *in vivo* imaging of liver was also achieved through tail vein injection of 2×10^{11} DNA particles of adenovirus carrying luciferase reporter [25]. Intriguingly, a similar time course of transgene expression was reported in liver using 10^9 pfu ($\cong 2 \times 10^{10} - 5 \times 10^{10}$ DNA particles) of adenovirus-firefly luciferase vector [26]. In contrast, infusion of higher viral particle numbers (2×10^{12}) resulted in a strong systemic inflammatory reaction that was very severe and sometimes fatal [27, 28]. The limited duration of fluorescent signal emission can be explained by previous reports that demonstrated the transient nature of adenovirus-mediated transgene expression was due to an immune response both to the vector and vector-infected host cells. This is considered to be a major obstacle limiting the therapeutic efficacy of adenoviral vector in the treatment of inherited diseases. However, in terms of mounting an immune response to tumor cells, the antigenicity of adenoviral vectors has been considered advantageous in clinical cancer gene therapy trials [29]. As reported previously, adenovirus-directed transgene expression usually decreases to undetectable levels within 2 to 3 weeks [30]. Nevertheless, in our study, adenovirus-derived EGFP expression was detectable over 30 days using non-invasive fluorescence imaging.

Ex vivo analysis of major organs dissected from BALB/c mice injected systemically with AdEGFP demonstrated that only the liver produced significant levels of fluorescent signals compared to other organs. It is well known that liver is very permissive to adenovirus infection [31], and most of the adenovirus vector ends up in liver after i.v. delivery through tail vein. Apart from the liver; spleen, lungs, heart, and kidneys are transduced poorly by adenovirus vectors, implying the existence of anatomical barriers to adenovirus infection [32] or decreased CAR expression. Similarly, our *ex vivo* CCD analysis of major organs demonstrated that this method could not detect EGFP expression in the other abdominal organs. Furthermore, i.v. injection of AdEGFP resulted in homogenous and diffuse EGFP expression in the liver. When the liver lobes were individually analyzed, EGFP expression was spread throughout the entire liver, similar to previous reports [25]. This may be advantageous for

some clinical gene therapy applications when transgene expression in the whole liver is desirable.

It is necessary to acknowledge that there are some drawbacks to fluorescence optical imaging. The use of external illumination results in light absorption and emission by endogenous tissue fluorophore. This autofluorescence causes high background noise masking-specific EGFP fluorescence [22]. In our experiments, gall bladder, urinary bladder, intestines, and stomach displayed autofluorescence [33, 34]. In addition, it is known that skin hair can also generate an autofluorescent signal [11]. Even after hair removal, fluorescence imaging of deep tissues can be difficult to perform due to the absorption and scattering of visible light by the living tissue itself. *Ex vivo* analysis of dissected organs and exposing them directly to camera resulted in a 36% increase in signal intensity. This is in accordance with previous studies, suggesting that the intensity of light is dependent on the depth of the transfected cells [33]. Using red fluorescent proteins that excite and emit light at higher wavelengths can partly overcome reduced light scattering and tissue obstruction. Accordingly, red fluorescent variants can be used to image deeper tissue. Moreover, red fluorescent proteins substantially reduce autofluorescence [35].

In conclusion, we have clearly demonstrated that *in vivo* non-invasive imaging using a CCD camera is a feasible option to detect adenovirus-mediated EGFP expression in live animals. In addition, systemic delivery of adenovirus vectors resulted in signal accumulation exclusively in liver. The procedure itself is a simple, cost-effective, and applicable method. The use of other gene therapy vectors, other fluorescent markers, and even other routes of gene delivery will certainly increase the application of this non-invasive fluorescent imaging technique.

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