Far-red fluorescence gene reporter tomography for determination of placement and viability of cell-based gene therapies

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Abstract: Non-invasive injectable cellular therapeutic strategies based on sustained delivery of physiological levels of BMP-2 for spinal fusion are emerging as promising alternatives, which could provide sufficient fusion without the associated surgical risks. However, these injectable therapies are dependent on bone formation occurring only at the specific target region. In this study, we developed and deployed fluorescence gene reporter tomography (FGRT) to provide information on in vivo cell localization and viability. This information is sought to confirm the ideal placement of the materials with respect to the area where early bone reaction is required, ultimately providing three dimensional data about the future fusion. However, because almost all conventional fluorescence gene reporters require visible excitation wavelengths, current in vivo imaging of fluorescent proteins is limited by high tissue absorption and confounding autofluorescence. We previously administered fibroblasts engineered to produce BMP-2, but is difficult to determine 3-D information of placement prior to bone formation. Herein we used the far-red fluorescence gene reporter, IFP1.4 to report the position and viability of fibroblasts and developed 3-D tomography to provide placement information. A custom small animal, far-red fluorescence tomography system integrated into a commercial CT scanner was used to assess IFP1.4 fluorescence and to demark 3-D placement of encapsulated fibroblasts with respect to the vertebrae and early bone formation as assessed from CT. The results from three experiments showed that the placement of the materials within the spine could be detected. This work shows that in vivo fluorescence gene reporter tomography of cell-based gene therapy is feasible and could help guide cell-based therapies in preclinical models.

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References and links

1. Introduction

Spinal fusion is a conventional therapeutic method to reduce pain arising from abnormal motion of the vertebrae by joining two or more vertebrae with a bone substitute [1].
procedure is usually performed in the lumbar spine area but is also used to treat diseases in the cervical and thoracic spine regions [2]. Spinal fusion has been developed with the bone substitutes conventionally made of ceramics [3] and acquired from the donors (allograft) or from patients (autograft) [4], and more recently, made of materials generated by molecular biological methods [1]. The latter includes osteoinductive growth factors such as bone morphogenetic proteins (BMPs) [5, 6], bone healing enhanced growth factors, and mesenchymal stem cells harvested from bone marrow [7, 8]. Among the molecular biological methods, BMP-based therapeutic strategies have become popular because of their high fusion rate and ease of applicability [2].

BMP-2 was first used for bone grafting with demineralized bone matrices (DBMs) which are generated by removing the inorganic mineral and retaining the organic collagen matrix [9]. Optimal treatment requires stable, high local concentrations of BMP-2 [10–12]. Recent case reports suggest that high doses of BMP-2 leads to rapid unwanted events including osteolysis of the bone and is potentially carcinogenic [13]. In vivo gene therapy is a viable effective alternative since it provides similar efficacy through delivery of sustained BMP-2 expression at 100-1000 fold lower concentrations. However, direct injection of viral carriers to produce BMP-2 in the body can lead to virus uptake in other tissues than the targeted area leading to potential serious complications. Further, target regions often have limited to no tropism for the viruses, resulting in poor BMP-2 expression at the target site [2]. Techniques that involve ex vivo transduction of cells with BMP-2 viral vectors circumvent these problems and allow for efficient expression of the BMP-2 at the site specific area. However, the cell based strategy depends on stable cell placement at the site where therapeutic action is needed [12, 14]. Recently, we showed that poly(ethylene glycol) diacrylate (PEGDA) hydrogel is an effective carrier for encapsulation of BMP-2 producing cells enabling stable placement and allows for the use of allogenic cells, without launching an immune response that could otherwise limit BMP-2 local release [12]. Methods to longitudinally image the placement of PEGDA encapsulated BMP-2 producing cells are needed to predict cell viability and location of optimal ossification and fusion to show preclinical efficacy.

In vivo fluorescence molecular imaging has sufficient sensitivity to probe biological processes at the molecular and cellular levels by targeting specific proteins with specific probes or reporters. Fluorescence gene reporters have high specificity for target identification and can retain image-able signals with cell division, providing a unique opportunity to longitudinally monitor therapeutic response in one single mouse [15]. However, the high absorption and autofluorescence of tissues at the visible excitation wavelengths of these reporters limit non-invasive, in vivo fluorescence gene reporter imaging [16]. Recently, the emergence of far-red fluorescence gene reporters, such as IFP1.4 [17] and iRFP [18], promises significant improvement in the sensitivity of in vivo planar fluorescence imaging. Yet planar, 2-D imaging does not afford 3-D localization information, which is important to evaluate placement of encapsulated BMP-2 producing cells and predict therapeutic response [19]. Herein, we employ fluorescence tomography of the far-red fluorescence gene reporter, IFP1.4 to directly show that placement of cell based therapies can be monitored with far-red fluorescence gene reporter tomography (FGRT).

In the following, we first briefly review methods for producing IFP1.4 expressing fibroblasts and their encapsulation with PEGDA, and then describe the application of far-red fluorescence gene reporter tomography (FGRT) following the encapsulated/unencapsulated cell injection in the paraspinous muscle of mice. For this application, we developed a bi-modality (far-red fluorescence/computed tomography (CT)) imaging system by integrating fluorescence imaging components into a Siemens Inveon scanner. A linear high-order simplified spherical harmonics approximation reconstruction algorithm with fully parallel acceleration was devised to reconstruct 3-D images detailing the position of the IFP1.4 gene reporter from 2-D projection data. The reconstructed fluorescence tomography shows the effectiveness of far-red FGRT for predicting the effect of placement of cell-based therapy.
2. Methods

2.1 Cell based gene therapy

Human diploid fetal lung fibroblasts (MRC-5) (ATCC, Manassas, VA) were propagated in Dulbecco’s modified Eagle’s medium at 37°C. Replication-defective E1-E3 deleted first-generation human type 5 adenoviruses (Ad) containing cDNAs for BMP-2, IFP1.4 in the E1 region of the virus were used to transduce the cells as previously described [20]. The viruses were confirmed to be negative for replication competent adenovirus and particle to plaque forming unit (PFU) ratios were 1:10, 1:25, and 1:16 respectively. Briefly, viral transductions (5000 vp/cell) were done using Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum as describe [20].

5 x 10^6 transduced cells alone or cells encapsulated with poly(ethylene glycol) diacrylate (PEGDA) were used as previously described [12]. Three mice were intramuscularly injected with microencapsulated (N = 2) or unencapsulated (N = 1) transduced cells in the right paraspinous muscle of a mouse lumbar spine. In vivo fluorescence tomography and CT was performed 14-15 days after injection. Animal studies were performed in accordance with an Institutional Animal Care and Use Committee (IACUC)–approved protocols at Baylor College of Medicine and the University of Texas Health Science Center at Houston.

2.2 Bi-modality fluorescence imaging system

We previously made use of a Siemens Inveon CT gantry to realize multi-modality near-infrared fluorescence (NIRF)/ positron emission tomography (PET)/CT imaging system from time-dependent, frequency-domain measurements and also validated 3-D fluorescence tomography with a dual-labeled imaging agent [21]. Briefly, the system consists of a gain modulatable NIR sensitive intensifier optically coupled to a front-illuminated charge-coupled device (CCD) camera for detection and a modulatable NIR laser diode. Herein, we adapted system by removing the NIRF illumination source and emission filters and replaced them with a 690nm laser diode (Intense Inc., North Brunswick, NJ) and interference filters enabling tomography of IFP1.4. While the far-red gene reporter could be tomographically reconstructed using frequency-domain measurements made at 100MHz with the system, limited IFP1.4 signal required that we conducted measurements in the continuous wave (CW) mode by simply removing the RF signal.

2.3 Fluorescence gene reporter tomography (FGRT) algorithm

To simulate the photon propagation in tissues in small animals, the high-order simplified harmonics spherical (SP_3) is used to provide more accurate simulations compared to classic diffusion approximation (DA) [22–24]. We developed a fully parallel linear reconstruction algorithm for fluorescence gene reporter tomography [25]. Specifically, the SP_3 approximation in the CW mode is as follows [22–24]:

\[
\begin{align*}
\frac{-1}{3\mu_a^{m,m}} & \nabla \phi_1^{m,m} + \mu_a^{m,m} \phi_1^{m,m} - \left\{ \frac{2}{3} \mu_a^{m,m} \right\} \phi_2^{m,m} = \left[ Q \mu_e^{\phi} \phi^{m,m} \right] \\
\frac{-2}{3} \mu_a^{m,m} & \phi_1^{m,m} - \nabla \frac{1}{7\mu_a^{m,m}} \nabla \phi_2^{m,m} + \left( \frac{4}{9} \mu_a^{m,m} + \frac{5}{9} \mu_s^{m,m} \right) \phi_2^{m,m} = \left[ -\frac{2}{3} Q \mu_e^{\phi} \phi^{m,m} \right]
\end{align*}
\]

where $\phi_1^{m,m}$ and $\phi_2^{m,m}$ are the composite moments of the excitation and emission radiances; $\phi^\phi$ is the fluence of the excitation and is equal to $\phi_1^{m,m} - \frac{2}{3} \phi_2^{m,m}$; $\mu_a^{m,m} = \mu_a^{m,m} + \mu_s^{m,m}(1 - g^\phi)$, where $\mu_a^{m,m}$ and $\mu_s^{m,m}$ are the absorption and scattering coefficients of the tissue at excitation and emission.
wavelengths, respectively; $g$ is the anisotropic factor; and $j = 1, 2, 3$; $\mu_i^f$ and $Q$ are the absorption coefficient and quantum efficiency of the fluorophore respectively; and $[\cdot]^*$ denotes the terms only for prediction of emission fluence. The mismatch of the refractive indices between the tissue and the external medium is considered using the corresponding boundary conditions

\[
\left\{ \begin{array}{l}
\left(\frac{1 + B_i}{3\mu_i^f}\right)\nu \cdot \nabla \phi_1^m = -\left(\frac{D_2}{\mu_2^f}\right)\nu \cdot \nabla \phi_2^m = -\left(1 + A_i\right)\phi_1^m + \left(1 + C_i\right)\phi_2^m \\
+ \int_{s_\infty} S(s) [i\cdot\nu]ds \\
-\left(\frac{D_2}{\mu_2^f}\right)\nu \cdot \nabla \phi_2^m = \int_{s_\infty} S(s) [i\cdot\nu]ds \\
\end{array} \right.
\]

where $A_1, \ldots, D_1, A_2, \ldots, D_2$ can be found in [22]; $\nu$ is the unit outer normal of the boundary; $\hat{s}$ is the incoming direction of the excitation light; $S$ is the excitation modulation light; and $[\cdot]^*$ represents the term used for describing excitation fluence. The measurable exiting partial current $J^{*,m,b}$ for emission is given as

\[
J^{*,m,b} = \frac{1}{4} + J_0 \left(\phi_1^m - \frac{2}{3} \phi_2^m\right) - \frac{0.5 + J_1}{3\mu_i^f} \nu \cdot \nabla \phi_2^m \\
+ \frac{1}{3} \left(\frac{5}{16} + J_2\right) \phi_2^m - \left(\frac{J_3}{7\mu_i^f}\right) \nu \cdot \nabla \phi_2^m
\]

where the coefficients $J_0, \ldots, J_3$ are found elsewhere [22]. With the finite element methods, we obtain the solution of $\phi_2^m$ using Eqs. (1) and (2). Using the method described in [25], for the emission equation, we have

\[
\begin{bmatrix}
M_{1\phi_1} & M_{1\phi_2} \\
M_{2\phi_1} & M_{2\phi_2}
\end{bmatrix}
\begin{bmatrix}
\phi_1^m \\
\phi_2^m
\end{bmatrix}
= \begin{bmatrix}
B^m \\
-\frac{2}{3} B^m
\end{bmatrix}
\]

where $M_{ik}$ are sub-matrices and can be computed using the finite element method [26]; $B^m$ is obtained by its components $b^m_{pq}$ and given as

\[
b^m_{pq} = \int_\Omega Q \phi^m \cdot v_p v_q dr
\]

where $v_p$ are the shape functions. By inverting the matrix on the left-hand side of Eq. (4) using similar method in [26], we have

\[
\begin{bmatrix}
\phi_1^m \\
\phi_2^m
\end{bmatrix}
= \begin{bmatrix}
IM_{1\phi_1} - \frac{2}{3} IM_{1\phi_2} \\
IM_{2\phi_1} - \frac{2}{3} IM_{2\phi_2}
\end{bmatrix} B^m \mu_i^f
\]

where $\mu_i^f$ are the absorption coefficients.
where $IM_{10}^j$ are the sub-matrices of the whole inverse matrix corresponding to $M_{10}^j$. After we remove the rows in matrices 
\[
\left(IM_{10}^j - \frac{2}{3} IM_{10}^{20}\right)B^w \quad \text{and} \quad \left(IM_{20}^j - \frac{2}{3} IM_{20}^{20}\right)B^w
\]
corresponding to the non-boundary measurable discretized points. We can use Eq. (3) to obtain
\[
J^{x,m,b} = \beta_1 \phi_{1m}^{x,b} + \beta_2 \phi_{2m}^{x,b} = \left(\beta_1 G_1 + \beta_2 G_2\right) \mu^f_{sf} = G \mu^f_{sf} \quad \text{(7)}
\]
where $\beta_1$ and $\beta_2$ can be obtained using Eq. (2); $G_1$ and $G_2$ are the corresponding matrices after the operation of row removing in Eq. (6); and $G$ is the relationship matrix between the unknown $\mu^f_{sf}$ and the acquirable measurements $J^{x,m,b}$. When there are $N_v$ different illuminations at different positions, we have
\[
J^{x,m,b} = A \mu^f_{sf} \quad \text{(8)}
\]
where
\[
J^{x,m,b}_T = \begin{bmatrix}
J^{x,m,b}_1 \\
\vdots \\
J^{x,m,b}_v \\
\end{bmatrix}, \quad A = \begin{bmatrix}
G_1 \\
\vdots \\
G_v \\
\end{bmatrix}
\quad \text{(9)}
\]
Finally, we can use the limited memory variable metric-bound constrained quasi-Newton method (BLMVM) to solve the following least squares problem for FGRT:
\[
\min_{\mu^f_{sf} < \mu^f_{sf}^{\text{sup}}} \theta(\mu^f_{sf}) : \left\| A \mu^f_{sf} - J^{x,m,b}_T \right\|^2 \quad \text{(10)}
\]
where $\mu^f_{sf}^{\text{sup}}$ is the upper bound of physical constraint of $\mu^f_{sf}$. It is noteworthy that the regularization terms are not used in the algorithm.

2.4 Small animal tomography

Mice were first imaged with CT scanner in low spatial resolution mode (with voxel length of 0.1mm) to provide whole-body surface information for registration of fluorescence measurements and tomographic reconstruction. CT scanning was then performed with high spatial resolution mode in the lumbar spine region (with voxel length of 0.06mm) in order to acquire better CT images for localizing cell placement. For fluorescence imaging, planar images of fluorescent photon distribution on the animal surface following transillumination of excitation light were acquired by rotating the optical/CT gantry to 4 different angles ($0^\circ$, $45^\circ$, $180^\circ$, and $315^\circ$). For each projection, we used an aspheric lens to shape the laser to form a ~1 mm diameter point on the tissue surface. Fluorescence image integration times were varied between 300 and 1600 ms to obtain total photon count rates of approximately 40,000 and five separate images at each projection were acquired to further improve the signal-to-noise ratio (SNR) without oversaturating the limited dynamic range of the 16-bit CCD. After imaging was completed, the mice were sacrificed. The tissues of lumbar spine were extracted and were further scanned by CT with high spatial resolution mode to confirm the imaging information from in vivo CT images.

In order to reconstruct the far-red fluorescence tomograms from the acquired fluorescent photon distribution on the animal surface, a volumetric mesh representing the truncal region...
(and spine) was generated from tissue surface boundaries obtained from the low resolution CT image. Image segmentation, surface mesh generation and simplification, and tetrahedral volumetric mesh generation were performed using the commercial software Amira 5.0 (Visualization Sciences Group, Burlington, MA). The meshes had an average element diameter of 1.0mm and the number of the discretized points ranged between 13,000 and 24,000. Using linear reconstruction algorithms, the reconstruction was performed on a cluster of eight nodes (eight CPU cores of 3.0 GHz and 16 GB RAM at each node) with input parameters of the mapped fluorescent photon distribution and uniform tissue optical properties of 0.057 mm\(^{-1}\) (absorption coefficient) and 8.50 mm\(^{-1}\) (scattering coefficient).

3. Results and discussions

3.1 Far red FGRT for spinal fusion

![Reconstructed gene reporter distribution in cross-sections](image.png)

Fig. 1. The reconstructed gene reporter distribution in the cross-sections with the maximal reconstructed values (the first, second and third rows). Top 80%, 90%, and 99% reconstructed values are shown, respectively. The fourth row shows the position of the cross-sections. “M1”, “M2”, and “M3” are Mouse 1, 2, and 3, respectively.

Figure 1 shows the axial cross-section indicating the location of the IFP1.4 gene reporter with the contour map representing the values of \(\mu_s^a\) thresholded to depict those values with 80%, 90%, and 99% of the maximum reconstructed values. Mouse 1 and 3 (M1 and M3) and 2...
(M2) were injected with microencapsulated and unencapsulated transduced cells respectively. Due to high scattering and absorption of fluorescent photons and limited spatial resolution of fluorescence tomography, it is generally difficult to reconstruct distributed interior objects, which may be reflected in the reconstructed results of unencapsulated cells implanted in M2. While all three reconstructed cross-sections show a reconstructed fluorescent target arising from cells located at the implantation site, the axial image contains significant interior artifacts when low threshold criteria are used in the animal implanted with cells not protected by encapsulation. Although more artifacts appear in M2 compared to M1 and M3, FGRT generates one bright spot to represent the site of cell implantation. Because M1 and M3 were injected with microencapsulated cells, similar reconstruction results were obtained. Interior artifacts probably arise due to the reduced SNR associated with the loss of far-red fluorescent signals and cell viability of unencapsulated cells. Surface artifacts that occur in the reconstructions of M1 and M3 are expected and routinely filtered from the image (The images shown in Fig. 1 were not processed).

3.2 Combined CT and FGRT images

Figure 2 shows new bone formation from high-resolution CT (the first column), co-registration of low resolution CT and FGRT (the second column). It is noteworthy that low resolution CT does not provide sufficient resolution for detection of early stage heterotopic bone ossification. While it is not possible to directly register high resolution CT images of heterotopic bone ossification with FGRT images derived from volumetric meshing constructed from low-resolution CT, the position of the IFP1.4 fluorescent targets co-locates in two (M1 and M2) of the three cases. In the third case with encapsulated cells, no evidence of heterotopic bone ossification was noted on high resolution CT. Due to the reduced production of BMP-2 in IFP1.4 infected fibroblasts, we could not directly correlate FGRT images with response to therapy in this initial study.

In all cases the reconstructed IFP1.4 target location corresponded to implantation site and was adjacent to vertebra in the spine. In the cases of encapsulated cell (M1, M3) and unencapsulated cell (M2) implantation in which heterotopic bone ossification occurred, mineral deposition was located between 2.2 and 2.6 mm away from the reconstructed IFP1.4 target (Table 1). (Since new bone imaging information of M3 could not be acquired from the CT images, we could not obtain quantitative compared information). The differences between fibroblast position (due to IFP1.4) and bone formation (from CT) could be explained by (i) the intrinsic localization error of fluorescence tomography, (ii) the inability to detect non-mineralized ossification tissues for accurate co-localization of BMP-2 expressing cells and heterotopic bone ossification. We have performed the imaging performance evaluation of the developed fluorescence tomography by using gold-standard nuclear imaging of dual-labeled imaging agents [21] and found a smaller, but similar difference (2.15mm) between the fluorescent reconstructed results and the PET validation imaging information. The intrinsic localization error of the fluorescence tomography could contribute to the positional mismatch of fibroblast placement and eventual bone formation.

| Table 1. Quantitative analysis of the CT and optical imaging information based on Fig. 2. “N.A.” is due to the absence of new bone imaging information. The distance errors were calculated regarding the center positions of the new bone in the CT and optical reconstruction. |
|------------------------|----------------|----------------|
|                        | Close to the same vertebra | Distance from the vertebra (CT mm) | Distance between CT and Optical (mm) |
| Mouse 1                | Yes                      | 0.90                                      | 2.20          |
| Mouse 2                | Yes                      | ~0.28                                     | 2.60          |
| Mouse 3                | N.A.                     | N.A.                                      | N.A.          |
Fig. 2. The first column shows that anatomical imaging information of new bone from BMP-2 using CT scanning with high spatial resolution mode. The imaging information of new bone for M3 cannot be acquired from the CT scanning. The second column is the reconstructed results from 3-D fluorescence gene reporter tomography. The arrows show the reconstructed new bone information. “M1”, “M2”, and “M3” are Mouse 1, 2, and 3, respectively.

4. Discussions and conclusion

By avoiding the high autofluorescence background that arises from visible excitation, we have for the first time, demonstrated 3-D tomography of fluorescence gene reporters to track localization information for cell- and gene-based therapy. Due to the unexpected suppression of BMP-2 production that arises in cells expressing IFP1.4, it is difficult to observe robust functional response to therapy. However, the tomographic results from both encapsulated and unencapsulated cell-based mouse experiments show the possibility and effectiveness of fluorescence gene reporter tomography. The influence of IFP1.4 expression on the production of BMP-2 needs further investigation, whether it be towards the development of new, more innocuous fluorescence gene reporters or better methods for co-infecting fluorescent protein reporters and therapeutic proteins. In addition, although the tomographic results are promising, we assumed the tissue optical properties are homogeneous, which potentially affected the localization precision. Conventional CT scanning has low imaging contrast especially for soft tissues. We will further investigate the effect of CT contrast imaging agent in cell-based spinal fusion. The suitable one will be used to improve CT image contrast and to improve the imaging quality of fluorescence tomography. Furthermore, the lack of brightness of IFP1.4 could create anomalies associated with changing tissue absorption and scattering properties. The deployment of time-dependent FDPM measurements of image reconstruction using second generation, far-red fluorescence gene reporters with higher fluorescent yields.
and less intrusive impact on expression of BMP-2 could improve the ability to correlate cell-
and gene-based therapies with treatment results.

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