Primary ciliary dyskinesia assessment by means of optical flow analysis of phase-contrast microscopy images

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A B S T R A C T
Primary ciliary dyskinesia implies cilia with defective or total absence of motility, which may result in sinusitis, chronic bronchitis, bronchiectasis and male infertility. Diagnosis can be difficult and is based on an abnormal ciliary beat frequency (CBF) and beat pattern. In this paper, we present a method to determine CBF of isolated cells through the analysis of phase-contrast microscopy images, estimating cilia motion by means of an optical flow algorithm. After having analyzed 28 image sequences (14 with a normal beat pattern and 14 with a dyskinetic pattern), the normal group presented a CBF of 5.2 ± 1.6 Hz, while the dyskinetic patients presented a 1.9 ± 0.9 Hz CBF. The cutoff value to classify a dyskinetic specimen was set to 3.45 Hz (sensitivity 0.86, specificity 0.93). The presented methodology has provided excellent results to objectively diagnose PCD.

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1. Introduction

Primary ciliary dyskinesia (PCD) is an autosomal recessive inherited disorder affecting approximately 1:10,000 to 1:30,000 individuals [1–3]. It causes a defect in the action of the cilia lining the respiratory tract (lower and upper, sinuses, Eustachian tube, middle ear), Fallopian tube, cerebrospinal fluid tract and spermatozoid flagella. PCD is characterized by the complete absence of mucociliary clearance, leading to respiratory symptoms and signs typically present since birth and predisposing affected individuals to recurrent respiratory infections [4]. Approximately half of sufferers have situs inversus [5–7].

Motile cilia play a crucial role in clearing mucus and debris from the airways under normal conditions, as can be seen in patients with abnormal airway ciliary beating caused by primary ciliary dyskinesia [8,9]. Motile cilia also play a role in circulating spinal fluid in the ventricles of the brain, where abnormal ciliary beating has recently been linked to hydrocephalus and other developmental cerebral abnormalities [10,11].

Despite persistent symptoms, and often attendance at ear, nose, and throat and respiratory clinics, many patients with PCD are not diagnosed until later in life [12], by which time permanent lung damage has occurred [13]. Early and accurate diagnosis is important, because once made, lung function can be maintained with specialist respiratory care [14–16]. The diagnosis of PCD is traditionally made on the basis of a supportive clinical history and an abnormal ciliary beat frequency (CBF). The most commonly used techniques (the modified photodiode [17] or photomultiplier method [18]) to measure CBF use an indirect method and do not provide information on ciliary beat pattern. New high-resolution digital high speed video (DHSV) imaging has allowed the precise measurement of the beat pattern of cilia [19].

A commonly used method to estimate CBF using a DHSV has been explained in [20,21]. This method computes the Fast Fourier Transform (FFT) of the intensity signals in a window of 3 × 3 pixels centered on a selected pixel above the cilium. This technique does not consider the global movement of all cilia and local illumination changes can affect the results.

Other novel methods to compute the CBF are based on estimating the movement of pixels or regions in the images by using different techniques, as motion templates [22] or the Lucas–Kanade algorithm [23]. However, these methods are not valid when the cell is moving due to its own ciliary beat.
The microscope was connected to a CCD camera (Digital Quad High Speed Progressive Scan Camera, JAI CV-A33 CL, Jai UK Ltd., Uxbridge, United Kingdom) that records the images with a matrix size of 649 × 494 pixels, and a rate of 120 frames per second.

Images were acquired into a HP Workstation xw6200 Xeon 3.4 GHz with 2 Gb of RAM system (Hewlett-Packard Company, Palo Alto, CA, USA) by means of an image acquisition board (NI PCIe-1429, Full Configuration Camera Link Image Acquisition, National Instruments, Austin, TX, USA).

### 2.2. Stabilization

Ciliated samples are placed in a liquid solution. Therefore, ciliary beat can cause a movement of the cells to analyze. In general, there are two groups: isolated cells that have a rotation movement, and cells that are stuck on the bottom of the slide and keep still. In the first case, it is necessary to eliminate this rotation movement to estimate CBF [25].

#### 2.2.1. Cell segmentation

For initiating the stabilization process, it is necessary to localize the cell to analyze. We propose a semi-automatic segmentation method based on gradient vector flow snakes to perform this task. A snake is an energy-minimizing spline guided by external constraint forces and influenced by image forces that pull it toward features such as lines and edges [26]. Instead of exploiting only image information as low-level edge-detection techniques do, snakes also use information about the boundaries as part of an optimization procedure. Snakes are active contour models: they lock onto nearby edges, localizing them accurately.

The active-contour model involves vertices connected by edge segments with, in general, two associated force terms. The internal force is computed based on the local shape of the contour. The external force (or image force) that drives the active contour to the boundary can be based on any conventional edge-detection technique. The internal and external forces may be weighted differently.

Mathematically, a snake can be defined in discrete form as a curve \( x(s) = (x(s), y(s)), s \in [0, 1] \) that moves through the spatial domain of an image to minimize the energy function

\[
E = \int_0^1 \left( \frac{1}{2} (\alpha |x'(s)|^2 + \beta |x''(s)|^2) + E_{int}(x(s)) \right) ds
\]

where \( \alpha \) and \( \beta \) are weighting parameters that control the active contour’s tension and rigidity respectively, and govern the effect of the derivatives of \( x(s) \). The external energy function \( E_{ext} \) is derived from the image so that it takes on its smaller values at the features of interest such as boundaries.

A snake that minimizes (1) must satisfy the Euler equation

\[
\alpha x''(s) - \beta x''(s) - \nabla E_{int} = 0
\]

where \( E_{int} = M(s) - \beta x''(s) \) and \( E_{ext} = -\nabla E_{ext} \) comprise the components of a force balance equation such that \( M + E_{ext} = 0 \).

Xu and Prince [27] proposed Gradient Vector Flow (GVF) to improve the capture range of the image force. GVF involves a vector field derived by solving a vector diffusion equation which diffuses the gradient vectors of a gray-level image. The solution for the GVF vector field involves a combination of Laplacian and gradient terms, and a blending factor is used for governing the trade-off between them. GVF snakes replace the potential force \( -\nabla E_{ext} \) by the gradient vector field. The GVF field can be defined as the vector field \( \nabla v(x, y) = (u(x, y), v(x, y)) \) that minimizes the energy function

\[
\hat{e} = \int \mu (u_x^2 + u_y^2 + v_x^2 + v_y^2) + |\nabla f|^2 |u - \nabla f|^2 dxdy
\]
where \( f(x, y) \) is an edge map derived from the image \( I(x, y) \), having the property that it is larger near image edges, and the parameter \( \mu \) is a regularization parameter governing the tradeoff between the first and the second term. This parameter should be set to higher values in noisy images and to lower values in normal images.

Using the calculus of variations [28], the GVF can be calculated by solving the next Euler equations

\[
\mu \nabla^2 u - (u - f_x)^2 + f_y^2 = 0 \\
\mu \nabla^2 v - (v - f_y)^2 + f_x^2 = 0.
\] (4)

The set of equations (4) can be solved by considering \( u \) and \( v \) as functions of time and computing

\[
u_t(x, y, t) = \mu \nabla^2 u(x, y, t) - (u(x, y, t) - f_x(x, y))^2 + f_y(x, y)^2
\]

\[
u_v(x, y, t) = \mu \nabla^2 v(x, y, t) - (v(x, y, t) - f_y(x, y))^2 + f_x(x, y)^2.
\] (5)

The steady-state solution \((t \to \infty)\) of these linear parabolic equations is the desired solution of the Euler equations (4). The equations in (5) are known as generalized diffusion equations. A stable explicit finite difference implementation for solving the steady-state solution of (5) was given in [29].

An example of GVF snake segmentation is given in Fig. 2. Control points used in the first frame of the sequence to initialize the algorithm are shown in Fig. 2(a). These points are selected as a first contour estimation. Subsequently, an iterative process is performed to adapt the snake to the cell contour; see Fig. 2(b). In Fig. 2(c), we can observe the final cell segmentation after 50 iterations.

In our method, a set of seed points is placed by the user as an initial contour estimation to segment the first frame. The segmentation of the first frame is used as a first contour estimation of the other sequence frames. Using this initial estimation, the algorithm computes an iterative process to perform the cell segmentation in each frame. In Fig. 2(d) and (e), we can observe the cell segmentation in different frames of a video sequence. Although the cell segmentation is not perfect, it is good enough to provide the necessary input to the rotation correction step to determine the angle that each frame forms with the first one.

2.2.2. Rotation correction

Segmented images are used to center the cell in the middle of the images. In this way, the centroid of the segmented region is situated in the center of the image. This point is considered as the rotation axis. Once the cell is centered, for each frame, the rotation is corrected by using a method based on Fourier–Mellin transforms [30]. The solution of this problem consists in finding the angle that each frame forms with the first frame of the sequence.

We consider a function of two variables in polar coordinates \( f(r, \theta), \forall (r, \theta) \in R^*_+ \cdot S^1 \) where \( r \) is the radial variable, \( \theta \) is the angular variable, \( R^*_+ \) is the set of strictly positive real numbers and \( S^1 \) is the unit circle in \( R^2 \).

For this function, the Analytic Fourier–Mellin Transform (AFMT) is defined as [31]

\[
M_{f_{\rho}(r, \theta)}(k, \nu) = \frac{1}{2\pi} \int_0^{2\pi} f(r, \theta) e^{-i\nu r} e^{-i\theta k} dr d\theta
\] (6)

where \( \sigma \) is a positive constant that prevents the divergence when \( r \to \infty \).

An interesting property of the AFMT is the translation theorem in log-polar coordinates. For two functions with \( g(r, \theta) = f(ar, \theta + \beta) \), this theorem is

\[
M_{g_{\rho}(r, \theta)}(k, \nu) = M_{f_{\rho}(a r, \theta + \beta)}(k, \nu) = a^{-\sigma} \nu^{-\sigma} e^{i\beta \nu} M_{f_{\rho}(r, \theta)}(k, \nu)
\] (7)

where \( \alpha \) and \( \beta \) are constants that represent scale and rotation changes, respectively.

On the other hand, we can define the following function

\[
E_{f_{\rho}(\rho, \nu)} = \left( \int_0^\infty \int_0^{2\pi} r^2 f(r, \theta) - g(r, \theta + \nu)^2 dr d\theta \right)^{\frac{1}{2}}.
\] (8)

This function measures, in all points of parameter space \((\rho, \nu)\), the similarity between both functions.

Applying Parseval’s equality [32] on (8), we obtain

\[
E_{f_{\rho}(\rho, \nu)} = \left( \int_0^\infty \sum_{k,z=2} |M_{g_{\rho}(r, \theta)}(k, \nu) - M_{f_{\rho}(r, \theta)}(k, \nu)|^2 dv \right)^\frac{1}{2}
\] (9)

Finally, applying the translation theorem (7), we have

\[
E_{f_{\rho}(\rho, \nu)} = \left( \int_0^\infty \sum_{k,z=2} |M_{g_{\rho}(r, \theta)}(k, \nu) - e^{iz\nu} M_{f_{\rho}(r, \theta)}(k, \nu)|^2 dv \right)^\frac{1}{2}
\] (10)

We now assume that there is not variation in scale \((\rho = 1)\). In this case, (10) yields

\[
E_{f_{\rho}(\rho, \nu)} = \left( \int_0^\infty \sum_{k,z=2} |M_{g_{\rho}(r, \theta)}(k, \nu) - e^{iz\nu} M_{f_{\rho}(r, \theta)}(k, \nu)|^2 dv \right)^\frac{1}{2}
\] (11)

If \( f(r, \theta) \) denotes the intensity function of an image and \( g(r, \theta) \) is the intensity function of the same image but rotated, the angle \( \varphi_{\text{opt}} \) that minimizes (11) is the angle that both images form. In Fig. 3, we can observe different frames of a moving cell video sequence with rotation correction.

An advantage of this method is that the calculation of the AFMT is very fast because, with a change of variables, it can be converted into a FFT.

2.3. Measurement of CBF

Once the movement of the cell has been eliminated, a region of interest (ROI) is selected by the user. This ROI permits to reduce the computational burden associated to the algorithm, reducing also the background noise, and thus providing more defined frequency peaks. This ROI includes all cilia and their movement along all the sequence. For the CBF analysis, a motion estimation technique based on optical flow has been implemented.

For computing the cilary motion, it is necessary to calculate the movement of the pixels between each frame and the initial frame. This motion has been computed by using the Farneback method [33], which uses polynomial expansion to approximate the neighbors of a pixel. This dense optical flow analysis produces a displacement field from two video frames.

The idea behind polynomial expansion is to approximate the image intensity in a neighborhood of each pixel with a set of polynomial functions. We are only interested in quadratic polynomials, giving us the local signal model, expressed in a local coordinate system,

\[
f(x) = x^TAx + b^T x + c
\] (12)

where \( A \) is a symmetric matrix, \( b \) a vector and \( c \) a scalar. The coefficients are estimated from a weighted least squares fit to the signal values in the neighborhood.

Following the description in Farneback and Westin [34], to derive the expression for the translation, we first consider a polynomial expansion of the signal, \( f_1 \), and a version of the signal that has been globally translated, \( f_2 \), by the vector \( d \).

\[
f_1(x) = x^TA_1 x + b_1^T x + c_1
\] (13)
Fig. 2. Cell segmentation process. (a) Selected control points on the original image, where the gradient vector flow has been superimposed on it. (b) Snake adapting to the contour cell, where the resulting contour segmentation corresponding to several iterations can be observed. (c) Cell segmentation – initial frame – (after 50 iterations); (d) Cell segmentation of the frame 100 of the video sequence; (e) Cell segmentation of the frame 200 of the video sequence.

\[
f_2(x) = f_1(x - d) = (x - d)^TA_1(x - d) + b_1^T(x - d) + c_1 = x^TA_1x + b_1^Tx + c_1
\]

Equating the coefficients in the quadratic polynomials yields

\[
A_2 = A_1 \tag{15}
\]

\[
b_2 = b_1 - 2A_1d \tag{16}
\]

\[
c_2 = d^TA_1d - b_1^Td + c_1 \tag{17}
\]

The key observation is that by equation (16) we can solve for the translation \(d\), at least if \(A_1\) is non-singular,

\[
d = -\frac{1}{2}A_1^{-1}(b_2 - b_1) \tag{18}
\]

We note that this observation holds for any signal dimensionality.

For motion analysis, we start by doing a polynomial expansion of both the left and the right images, giving us expansion coefficients \(A_l(x, y), b_l(x, y),\) and \(c_l(x, y)\) for the left image and \(A_r(x, y), b_r(x, y),\) and \(c_r(x, y)\) for the right image.

The first practical complication is that Eq. (15) assumes that the \(A\) matrix should be the same in both images.

As a practical solution, the arithmetic mean can be used,

\[
A(x, y) = \frac{A_l(x, y) + A_r(x, y)}{2} \tag{19}
\]
There are less difficulties with \( b \) and we can directly use \( b \), as \( b_1 \)
and \( b_2 \) as \( b_2 \). To simplify later steps of the algorithm we introduce

\[
\Delta b(x, y) = \frac{1}{2} (b(x, y) - b(x, y))
\]  

(20)

This turns Eqs. (16) and (18) into

\[
A(x, y)\Delta b(x, y) = \Delta b(x, y)
\]  

(21)

\[
d(x, y) = A(x, y)^{-1} \Delta b(x, y)
\]  

(22)

In principle, we should now be able to obtain a displacement vector
at \((x, y)\) from Eq. (22).

In Fig. 4(a), a ROI selection and an example of frame-by-frame
optical flow calculation are given. Optical flow computation
produces an individual displacement signal \( d_i(t) \) for each pixel \( i \) along
time. The approach used to estimate CBF computes the sum of the
FFT of these signals

\[
Y(f) = \frac{1}{NL} \sum_{i=1}^{N} |FFT(d_i(t))|
\]  

(23)

where \( N \) is the number of signals or number of pixels in the ROI and
\( L \) is the length of the signals or number of analyzed frames.

2.4. Regions under analysis and implemented methods

We have obtained the Farnebäck’s method based optical flow in
two different ways: either on the whole frame (Optical Flow on the
whole Frame or OF,Frame) or, in order to optimize the frequency
determination, on a region of interest focused on the ciliary zone of
the cell (Optical Flow over a specific Region of Interest or OF,ROI).
This ROI is manually selected by the user in the first frame of each
video sequence.

In order to compare the results obtained by applying the
Farnebäck’s method, we have also implemented the method pro-
posed in [20,21], consisting in estimating the CBF using a DHSV
through the computation of the FFT of the intensity signals in a win-
dow of \( 3 \times 3 \) pixels centered on a selected pixel above the cilia-
um (Int,S method).

2.5. Bland–Altman diagrams

Bland–Altman analysis [35] is a statistical method to com-
pare two different measurement techniques. In the Bland–Altman
graph, the differences between the two measurements are plot-
ted against the averages of them. Horizontal lines are drawn at the
mean difference, and at the limits of agreement, which are defined
as the mean difference \( \pm 1.96 \) times the standard deviation of the
differences.

2.6. Statistical analysis

Statistical analyses were performed with SPSS for Windows,
version 20 (IBM, Somers, NY, USA). After testing normal distri-
bution with the Kolmogorov–Smirnov test, t-Student test was
used to compare the two groups. Values of continuous variables
were expressed as mean \( \pm \) standard deviation. Receiver operating
characteristics (ROC) analysis was applied to evaluate the global
accuracy of ciliary beat frequency as a diagnostic tool. The per-
formance of the classifiers was evaluated by the area (Az)
and confidence interval at 95% (CI95%) under the ROC curve.

3. Results and discussion

In this section, we present experiments that apply the exposed
technique to images from real ciliary cells.

In order to perform cell segmentation, we have set \( \alpha = 1 \) and
\( \beta = 1.8 \) for the first frame, and \( \alpha = 0.5 \) and \( \beta = 2 \) for the other frames.

Our Farnebäck’s method based optical flow algorithm has been
tested on twenty-eight sequences: the normal group presented a
ciliary beat frequency of \( 5.2 \pm 1.6 \text{ Hz} \), while the dyskinetic group
presented a \( 1.9 \pm 0.9 \text{ Hz} \) ciliary beat frequency, \( t \)-Student \( p < 0.001 \).

Frequency as a diagnostic tool provided an \( Az 0.96 \) (CI95% 0.86–
1). The cutoff value to classify a dyskinetic specimen was \( <3.45 \text{ Hz} \)
(sensitivity 0.86, specificity 0.93).

As it can be observed in Fig. 4(b), the region of interest into which
perform the ciliary beat frequency analysis can have any shape, per-
mitting to perform an optimal decaramarck of the exact location of
the cells under study. The size of this ROI does not affect the algorithm accuracy, but it is important to isolate the background in order to reduce the existing noise. The computational burden associated to the algorithm is related to the size of the region under analysis. As a reference, for a ROI size of 26 × 30 pixels, the computational time is 40 s, while it is four times greater if the whole frame (155 × 118 pixels) is considered.

The gold standard for CBF estimation is the manual counting of the ciliary beats in a sequence of high speed video observed at slow motion (High Speed Visual Observation or HSVO). In this work, this task has been performed by a pulmonologist with 20 years of experience in the field.

In order to determine the performance of our method, we have compared the results obtained applying our method based in Farnebäck’s optical flow estimation on an specific region of interest focused in the ciliary zone of the cell (OF_ROI) (3.66 ± 2.25 Hz) (mean ± SD) to those obtained through visual observation on the same videos at slow motion (HSVO), (3.73 ± 2.20 Hz), and to a well established method consisting on the computation of the FFT of the intensity signals in a window of 3 × 3 pixels centered on a selected pixel in the cilium (Int_S method), (3.83 ± 2.10 Hz). We have also studied the influence of the region under study applying our method based in Farnebäck’s optical flow estimation on the whole frame (OF_Frame), (3.28 ± 2.15 Hz).

These data are graphically shown in the box-and-whisker plot of Fig. 5. This plot shows that the results of the OF_ROI method have less dispersion than those of the Int_S method. The latter method presents 5 outliers (near 20% of the measures), what confirms that it is less robust, being more affected by the experimental noise.

Table 1
Performance comparison with high speed video observed at slow motion (HSVO) of: OF_ROI (proposed optical flow method performed on the region of interest containing the ciliary zone); OF_Frame (optical flow method performed on the whole frame) and Int_S (intensity signal method).

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean difference</th>
<th>MSE</th>
<th>SD</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>OF_ROI</td>
<td>−0.06</td>
<td>0.04</td>
<td>0.20</td>
<td>−0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>OF_Frame</td>
<td>−0.45</td>
<td>0.30</td>
<td>1.65</td>
<td>−1.06</td>
<td>0.17</td>
</tr>
<tr>
<td>Int_S</td>
<td>0.10</td>
<td>0.12</td>
<td>0.63</td>
<td>−0.14</td>
<td>0.34</td>
</tr>
</tbody>
</table>

All units in Hz.
The *OF_Frame* method presents the highest dispersion, because it includes motion and noise outside the ciliary region.

Fig. 6 shows the Bland–Altman diagrams for the three considered methods. The 95% limits of agreement of *OF_ROI* method (−0.40, 0.20) are tighter than those of *IntS* method (−0.36, 1.30), whereas *OF_Frame* method presents the wider limits (−3.60, 1.70).

The results are summarized in Table 1, that shows the average, the mean square error (MSE) and the standard deviation (SD) of the differences between the measurements of the three considered methods and the HSVO method. We have also included the confidence interval (CI) of the mean with a CI of 95%. All these results confirm that our method (*OF_ROI*) provides more robust results than those obtained using the standard one, (*IntS*).

Examples of analyzed cells with *OF_ROI* method can be observed in Fig. 7. In Fig. 7(a), the CBF value is 6.0 Hz. We can observe other frequency components that correspond to harmonics of the fundamental frequency. Cilia of normal cells are characterized by a whip-like movement that corresponds to a sawtooth displacement signal and its spectrum contains both even and odd harmonics of the fundamental frequency.

In Fig. 7(b), after analyzing the sum of the FFTs, we obtain a CBF value of 3.4 Hz. In this sequence, a high noise level is present and harmonics are not visible. Despite the fact that all video sequences have been acquired with the same system, microscope focusing and illumination control is a manual process that can cause small variability in the different acquisitions. An improvement of this process should be developed in order to homogenize image acquisition.

The length of the video sequences is 590 frames corresponding to a frequency resolution of 0.1 Hz for the FFT calculation. A high frequency resolution is very important in pharmaceutical research and development of medicinal products for the treatment of CDF. The ability of this technique to detect small changes in CBF should make it an optimal method to evaluate how different compounds affect cilia motion.

4. Conclusions

In this paper, we present a system for analysis of motion in ciliary cells in order to estimate CBF. Studying cilia movement is a very challenging task because it is necessary to previously eliminate undesirable movements of the own cell. For this reason, we have implemented a stabilization process based on cell segmentation and Fourier–Mellin transforms. This technique provides a fast and robust method for eliminating rotations and permits to perform the CBF analysis precisely.

In this work, the cilia motion has been analyzed with a novel technique based on optical flow providing a very good performance to differentiate non-dyskinetic from dyskinetic cilia movement and motility. Compared to existing approaches in this field, our approach is a substantial improvement for studying the cilia movement. Conventional methods, which analyze the intensity variations of an isolated pixel, are not robust to noise and illumination changes due to the own cilia movement. These methods estimate CBF using local information. On the other hand, our method estimates CBF extracting the movement of all pixels that belong to cilia. This global information can also be used to obtain a complete pattern of movement and detect other possible abnormalities in the movement and characteristics of cilia.

This study provides an important improvement of the HSVF technique which allows performing CBF measures directly from biopsies of the suspected CDF patients in no more than 15 min without external altering factors. This fact permits an easy and immediate diagnosis of ciliary activity in situ avoiding complications and large time processing samples presents in other diagnostic tools.

The major limitation of the method is the use of manual process to perform some tasks: focusing, seed selection for initial segmentation and ROI initialization. In a future work, we want to work on managing the hardware to perform focusing automatically. We are also interested in developing algorithms to perform an automatic initialization based on movement detection.

The use of digital high speed video to estimate ciliary beat pattern and beat frequency is a powerful tool in the investigation of patients with primary ciliary dyskinesia. The next step in our investigation will analyze the effects that different drugs produce in CBF and study other ciliary abnormalities using global motion information obtained by the exposed method.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.compmedimag.2013.12.010.

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