INTRODUCTION:

Intercellular signaling is essential for the control of tissue differentiation in general. It is well known that growth factor signaling is of particular importance in developing bones, to control the process of endochondral ossification. In cartilaginous tissues, which lack direct cell-cell contact as well as vasculature, diffusion transport of nutrients and signaling molecules is obviously important.

Fluorescence recovery after photo bleaching (FRAP) is a generally used method to determine diffusivity. Available methods [1], however, assume homogeneity of tissues surrounding the bleach area and neglect geometrical restrictions to diffusion. Hence, diffusion coefficients in non-homogeneous materials, such as most biological tissues, cannot be assessed appropriately. This is illustrated in figure 1.

![Figure 1: Theoretical diffusion profiles after bleaching (dark spots) in the same tissue at different locations. In the top row ('without bone') the tissue surrounding the bleach spot is homogeneous. In the bottom row ('with bone') the bleach spot is close to a tissue which exhibits lower diffusivity (indicated with a grey box in the first image). The recovery profile between both situations differs, and so does the recovery of the mean intensity value within the bleached area with time (graph). Hence, if diffusivity was derived from the recovery curve, two different diffusion coefficients were ambiguously obtained for identical tissues.](image)

We developed a method which is suitable to determine diffusivity in non-homogeneous tissues from a time series of images obtained by FRAP. This method uses all available spatial information about tissue non-homogeneity as well as the complete recovery profile in the images, rather than the recovery curve off a selected part of the image only. In this way, diffusion coefficients in separate areas of the tissue can be obtained, based on a single FRAP measurement.

METHODS:

In the new procedure a FEA model, which accounts for Fick’s diffusion law, is used to simulate the diffusion process as observed in a time series of images. The concentration distribution in the first post-bleach image is fed back to the FEA mesh, by assigning the nearest pixel intensity value to each element (Fig 2, top row). In addition, areas which are expected to have distinct diffusion coefficients (e.g. different tissues, or cells within a tissue), are pre-defined in the mesh. This geometrical information is easily obtained from pre-bleach images.

Starting with the initial intensity distribution, and a first estimate for the diffusion coefficient per pre-defined zone, the diffusion process is simulated (Fig 2). Using an iterative least mean squares fitting procedure, minimizing the differences between the intensities in the pixels of the images and the elements in the FEA mesh, the diffusion coefficients for each pre-defined area is obtained.

For validation, this method was first applied to a time-series of 100 FRAP images from two homogeneous 3% agarose discs, loaded with 66 kDa fluorescein-albumin (Zeiss LSM510, bleach area radius 18 µm, 1 images*, 54*54 µm, 128*128 pixels). The diffusion coefficients in these particular discs were previously determined at 37.12 and 40.71 µm²/s, respectively [2,3].

The feasibility of the new procedure was shown by analysis of FRAP data from the proliferation zone of a growth plate (Fig 2). The tissue was bleached in-between two columns of cells, while different diffusion coefficients were fitted for the column of cells and the extracellular matrix, respectively.

RESULTS:

The diffusion coefficients for the agarose discs obtained with the new method were 36.44 and 39.54 µm²/s, consistent with those previously determined (37.12 and 40.71 µm²/s [2,3]), showing that the method is valid for determining diffusion coefficients from raw FRAP images directly.

From the FRAP measurements in the growth plate we derived diffusion coefficients of 49.22 µm²/s for the extracellular matrix and 3.87 µm²/s for the column of cells. With the same procedure, yet without accounting for tissue non-homogeneity, an incorrect diffusion coefficient of 19.75 µm²/s was computed, which illustrates the essence of the new approach.

DISCUSSION:

A method to evaluate FRAP data in non-homogeneous materials was developed and validated on homogeneous agarose gels with known diffusion coefficients. The applicability of the method to biological tissues was demonstrated in the proliferation zone of the growth plate, which contains columns of cells separated by extracellular matrix. To the best of our knowledge, this is the first method which accounts for non-homogeneities in the evaluation of FRAP images, while taking all available spatial information from the post-bleach images into account. It was shown that neglecting the non-homogeneities results in significant errors in the diffusion coefficients assessed.

In addition to non-homogeneities, the method inherently accounts for anisotropy and geometrical restrictions, while immobilized solute fractions are easily derived. This makes the method particularly suitable to evaluate diffusivity in biological tissues.

All information needed to assess diffusion coefficients in the tissue is included in the initial concentration profile, which is derived from the time-series of images. In this way the analysis becomes independent from the way in which a concentration gradient is applied. This is evaluated by successful analyses of FRAP data after bleaching of circular, square and rectangular areas (data not shown).

We conclude that we have developed a method that can be used to determine diffusion coefficients in non-homogeneous tissues, based on any time-series of diffusion images, which is well applicable to FRAP images. This method now enables determination of diffusivity of nutrients and signaling molecules in non-homogeneous tissues. Such data is essential to understand diffusion-dependent phenomena in biological tissues, such as nutrient supply in avascular tissues and long-distance signaling by growth factors.

REFERENCES:


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