JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 521–526

Received August 25, 2004
Accepted December 20, 2004
DOI 10.1211/0022357055777
ISSN 0022-3573

High-speed digital imaging method for ciliary beat frequency measurement

Svetlana Dimova, Frederik Maes, Marcus E. Brewster, Mark Jorissen, Mark Noppe and Patrick Augustijns

Abstract

The aim of this study was to develop a high-speed digital imaging system and related software for ciliary beat frequency (CFB) analysis in order to establish an automated and reliable method that is observer independent and faster compared to the conventional computerized microscope photometry method. Using primary human nasal epithelial cell cultures, the CBF was recorded with a computerized microscope photometry system and a high-speed digital imaging system. To obtain a wide range of frequencies, glycocholate (0.5%) and chlorocresol (0.005%) were used as cilio-stimulatory and cilio-inhibitory reference compounds, respectively. The mean values in hertz ($\pm$ s.d.) obtained with the photometry and high-speed digital imaging systems were: controls 8.2 ± 0.9 and 7.9 ± 1.1; chlorocresol 5.0 ± 0.9 and 5.1 ± 1.1; glycocholate 9.8 ± 1.0 and 9.7 ± 0.8. A similar increase (by 20 and 24%) and decrease (by 38 and 35%) in CBF was determined by the two methods after glycocholate and chlorocresol treatment, respectively. The mean difference between the photometry and high-speed digital imaging methods was 0.2 ± 0.6 Hz, and the Bland–Altman limits of agreement were from -1.0 to $+1.4$ Hz, suggesting that the results obtained by these two methods could be used interchangeably. These results show the reliability of the high-speed digital imaging system and the software developed for in-vitro CBF measurements. The advantages of the system include: (i) fast data acquisition and calculation, (ii) whole field automated CBF analysis and (iii) reduction in selection bias.

Johnson & Johnson – Pharmaceutical Research and Development, A Division of Janssen Pharmaceutica NV, Beerse, Belgium

Svetlana Dimova, Marcus E. Brewster, Mark Noppe

Department of Electrical Engineering (ESAT/PSI) and Radiology, Katholieke Universiteit Leuven, Leuven, Belgium

Frederik Maes

Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven, Leuven, Belgium

Svetlana Dimova, Patrick Augustijns

Laboratory for Experimental Otorhinolaryngology, Katholieke Universiteit Leuven, Leuven, Belgium

Mark Jorissen

Correspondence: Patrick Augustijns, Laboratory for Pharmacotechnology and Biopharmacy, Katholieke Universiteit Leuven, Herestraat 49, O&N, 3000 Leuven, Belgium. E-mail: patrick.augustijns@pharm.kuleuven.ac.be

Acknowledgement: This work was financed by the J&J Innovation Fund.

Introduction

For drug delivery, the nasal route is attractive, non-invasive and an alternative to the oral route. This is particularly important for drugs that are chemically and metabolically unstable in the gastrointestinal tract. At an early stage in drug discovery and during the development process, it is essential to gain a thorough insight of the nasal absorption potential, metabolism and toxicity of the active compound and formulation components.

A prerequisite for nasally applied formulations is that drugs and additives in the dosage forms do not interfere with normal nasal functioning such as nasal mucociliary clearance. Ciliary beat frequency (CBF) is one of the basic parameters of ciliary function and is generally accepted as the most relevant one. Different methods can be used to estimate CBF. A variety of traceable compounds, including saccharin and indigo carmine, have been used to estimate the mucociliary transport rate as an indication of CBF. The first measurement of ciliary beat frequency dates back to 1844 and was performed by Martinus, who used a stroboscope to estimate CBF. However, this method appeared to be unreliable (Lale et al 1998). During the last few decades more standardized and robust techniques have been developed. The technique most frequently used to measure CBF is the photo-electric method (Rutland & Cole 1980; Deitmer 1986; Jorissen & Bessems 1995) in which changes in light intensity induced by beating cilia are recorded by a photosensitive cell. The signal is subsequently converted into an electric current. After digitization, the CBF can be calculated using fast Fourier transformation (FFT) (Philips et al 1990). Although reliable and reproducible, this technique is laborious, time-consuming and prone to selection bias.

CBF data can also be obtained by monitoring ciliary beating using a video camera or a digital high-speed video system, followed by off-line analysis either using manual counting of the ciliary beats on replaying the pictures over defined time intervals (Rautiainen et al 1992; Chilvers & O'Callaghan 2000; Doran et al 2004) or by analysing changes in image intensity at defined locations (Nasr et al 1995; Sanderson 2000). The use of standard video camera equipment induces a clinically relevant upper limit of measurable frequencies, while the use of a high-speed camera is time-consuming and reduces the number of cilia measured in one sample.

To overcome a number of these problems, a high-speed digital system and dedicated software for in-vitro CBF analysis have been developed, evaluated and presented in this paper.

We validated our experimental whole-field high-speed digital imaging technique against the established photometry technique for measuring subtle effects on CBF (in the order of 2–3 Hz) induced by pharmaceutical excipients. Our results show that both techniques yield highly consistent results, with changes in CBF as measured with one technique being almost identically reproduced with the other.

Materials and Methods

Chemicals and materials

Pronase XIV, glycocholate and penicillin–streptomycin solution (10 000 IU mL^{-1} and 10 000 $\mu g\,mL^{-1}$, respectively) were purchased from Sigma Chemical Co., Ltd (St Louis, MO). DMEM–Ham's F12 1:1 medium, Ultroser G and NU-serum were obtained from Life Technologies Ltd (Paisley, UK). Vitrogen was purchased from Nutacon BV (Leimuiden, The Netherlands), HEPES buffer from Cambrex Bio Science Verviers sprl (Verviers, Belgium), chlorocresol from UCB (Leuven, Belgium) and polyethylene terephthalate membrane with pore size of 3 μm from Cyclopore SA (Louvain-La-Neuve, Belgium). The MinuCell perfusion system container (24 × 13 mm rings), 13 mm diameter cell-support material holding rings, a thermo plate (Medax, Nogel GmbH) and an IPC high-precision multichannel dispenser (Ismatec SA, Labortechnik-Analytik, Glattbrugg-Zürich, Switzerland) were obtained from Minucells and Minutissue Vertriebs GmbH (Bad Abbach, Germany). Tissue culture six-well plates (Nunc) were obtained from International Medical Products (Brussels, Belgium), 12-well plates (Costar) from Elscolab (Kruibeke, Belgium), 25 cm^2 tissue culture flasks and a 70 μm nylon cell strainer from BD Falcon (Oxnard, CA).

Cell isolation and culture

Human nasal epithelial cells were obtained by protease digestion of surgical specimens of nasal epithelial tissue as described previously (Dimova et al 2003). Briefly, the human nasal epithelial tissues were enzymatically dissociated using 0.1% pronase solution in DMEM–Ham's F12 1:1 medium, supplemented with 50 IU mL^{-1} penicillin and 50 $\mu g\,mL^{-1}$ streptomycin for a period of 24–44 h at 4°C. The protease activity was inhibited with 10% NU-serum. The cells were washed three times in DMEM–Ham's F12 1:1 medium, supplemented with 50 IU mL^{-1} penicillin, 50 $\mu g\,mL^{-1}$ streptomycin and 2% Ultroser G, by centrifugation (800 rpm, 5 min, 4°C) and incubated for 1 h in a 25 cm^2 plastic tissue culture flask in a CO_2 incubator (5% CO_2/95% air, 37°C) to allow selective attachment of the contaminating fibroblasts and macrophages. The final cell suspension was filtered through a 70 μm pore size nylon filter. The cell viability was assessed by trypan blue exclusion.

The cells were plated at a density of 0.8–1.0 × 10^6 viable cells cm^{-2} on polyethylene terephthalate membranes (3 μm pore size), fixed in 13 mm MinuCell holding rings and coated with glutaraldehyde-stabilized Vitrogen (1.2 mg mL^{-1}). The cells were initially cultured in DMEM–Ham's F12 1:1 medium, supplemented with 50 IU mL^{-1} penicillin, 50 $\mu g\,mL^{-1}$ streptomycin, 2% Ultroser G and 10 ng mL^{-1} cholera toxin in a CO_2 incubator. The cultures were transferred from the CO_2 incubator into the perfusion system container on day 8 after plating. The container was placed on a 40°C thermo plate with a cover lid. DMEM–Ham's F12 1:1 medium, supplemented with 100 IU mL^{-1} penicillin, 100 $\mu g\,mL^{-1}$ streptomycin, 2% Ultroser G and 20 mM HEPES (35 mM final concentration) was continuously perfused at a rate of 30 $\mu L\,min^{-1}$. Human nasal epithelial cells were cultured at perfusion system conditions for 7 or 14 days.

Approval was granted by the institutional Committee for Medical Ethics and Clinical Research.

Cell treatment

Glycocholate (0.5%) and chlorocresol (0.005%) have been used as cilio-stimulatory and cilio-inhibitory compounds, respectively. The cells were preconditioned for at least 30 min at 24°C. CBF was measured between 10 and 30 min after exposure to the test compounds (2 mL $well^{-1}$, 12-well plates). To investigate the reversibility of the cilio-stimulatory and cilio-inhibitory effect of glycocholate and chlorocresol, the CBF was also determined after withdrawal of xenobiotic exposure (between 25 and 45 min after rinsing of the cells (3 × 2 mL medium in 3 min) and incubation with medium). The control cells were incubated with medium only and treated in the same way. Because no differences were observed in the control CBF values and in the reactivity of the cilia between day 7 and 14 at perfusion system conditions, the results obtained at these time points are presented together.

CBF measurements

The CBF was determined at 24.1 ± 0.2°C by a computerized microscope photometry system and a high-speed digital imaging system, supplied by Omnilabo, Belgium. An inverted microscope (Olympus IX70) was used for the records made by the two systems at a magnification of 600×.

Computerized microscope photometry method
The signal from the fluctuations of light intensity, caused by beating cilia, was transduced to an electrical signal, amplified and transmitted to a personal computer using the FluarQuant photometry system (Applied Scientific Instrumentation, Inc., Eugene, OR). The signal was measured for a period of 1 min, with a sampling interval of 5 ms (200 samples per second) (FluarQuant software running under Windows 2000). The recorded signal was analysed by performing time spectral analysis using FFT on the waveform obtained (WinDaq software). From the signal obtained for 1 min, we analysed each cell for 10 periods, each 5 s long. The frequency corresponding to the highest peak of the first harmonic within these time segments was recorded as the CBF value within the analysed region of interest (ROI).

High speed digital imaging method
Data acquisition. A *Motion*Scope high-speed digital camera and PCI application software, running in a Windows 2000 environment (Redlake MASD Inc., San Diego, CA), were used for image acquisition. The images were captured at a frame rate of 500 frames per second (FPS) with a sampling interval of 2 ms. A sequence of 1024 images was recorded for each area for approximately 2 s. Each sequence of frame-by-frame images was stored in a file folder containing 1024 TIF format files for later retrieval and analysis. The resolution of the obtained images at a microscope magnification of 600× and a sampling rate of 500 frames per second was $320 \times 280 \times 8$ pixels, where one pixel corresponds to 0.1277 μm.

Calculation of CBF. In our approach, a CBF value is computed locally for each pixel separately by spectral analysis of the variation of the pixel intensity over time. First, an ROI is selected using an automated procedure such that stationary image regions, where no apparent motion is observed, are excluded from the analysis. This ROI is defined to include all pixels for which the standard deviation of the intensity variation (SDIV) over time exceeds a certain threshold value. This threshold is, by default, set at a signal-to-noise (SNR) ratio of 5 dB above the SDIV of stationary pixels, where the intensity variation is assumed to be due to imaging noise only. We define this noise-only SDIV value as the mode (i.e. the most frequent value) of the SDIV values of all pixels in the image as obtained by histogram binning. The percentage motile area was also calculated (the area of the selected motile ROI (in pixels) divided by the total area of the image (in pixels)). The ROI can be adjusted interactively, if required, by changing the SNR threshold value. For each pixel in the ROI, the influence of noise on the CBF computation is reduced by spatial averaging of the intensity signal at each time point within a 3×3 pixel region centred around that pixel. FFT analysis is then applied to the smoothed intensity signal. With typically $Nt = 1024$ time frames acquired at $FPS = 500$ frames per second, FFT analysis results in $Nf = (Nt/2) + 1 = 513$ independent FFT amplitude values at frequency steps $df = FPS/Nt = 0.4883$ Hz in the range 0 Hz to $f_{max} = (Nf - 1)*df = FPS/2 = 250$ Hz. The CBF is then computed as the frequency corresponding to the maximal FFT amplitude value in the range 0 to 20 Hz, assuming that the frequency responses at frequencies higher than 20 Hz are due to noise or higher harmonics of CBF. The CBF values, so computed for each pixel within the ROI, can be visualized as an image and inspected jointly with the original video images using a coupled cursor display. Individual areas (3×3 pixels) can thus be selected in the video images and their CBF can be obtained from the CBF image. Alternatively, features of interest in the CBF or associated amplitude image can be immediately located in the original video images. The limitations of this approach lie in the selection by the operator of the analysed regions and the time needed for the analysis of one record. Histogram analysis of the CBF image allows deriving of overall statistics (mean, standard deviation, median) for the CBF of all beating cilia in the image. The impact of different conditions on CBF can be easily visualized by comparing the CBF histograms obtained for each case. The histogram approach also made possible the filtration of double high frequencies and vibration-related low frequencies (1–2 Hz) applying a limiting right and left scale to the histogram map. Using this approach: (i) the region selection bias was avoided, (ii) the calculation time was reduced and (iii) the reproducibility of the results obtained from one record on repeated calculation(s) was increased. Because of these advantages, the histogram approach was used in this study. The data analysis was based on the mean CBF value from each image. The analysis method was implemented in Matlab (The Mathworks, Inc., Natick, MA) and a graphical user interface was developed for CBF measurement and histogram analysis.

Data presentation and statistical analysis

The results are expressed in hertz as mean ± s.d. Comparisons were made using paired Student's *t*-test. The level of significance was considered to be $P < 0.05$. The agreement between two CBF analysis methods was determined using the method suggested by Bland & Altman (1986). The Bland–Altman limits of agreement were calculated from the mean difference between the values obtained by the photometry and high-speed camera system ± twice the standard deviation.

Results and Discussion

Human nasal epithelial cells cultured on Vitrogen-coated polyethylene terephthalate membranes, under perfusion system conditions (Minucell and Minutissue) were used in this study. This cell-culture system offers the possibility for long-term preservation (up to 23 days after plating, 14 days under perfusion system) of well-differentiated ciliated cells with stable and reactive ciliary activity in-vitro. The mean

control CBF value, determined by the computerized microscope photometry method, at 24.1 ± 0.8°C was 8.6 ± 0.7 Hz (Dimova et al 2004). The main goals of this study were: (i) to validate the high-speed digital imaging method for CBF measurements and (ii) to test its potential for cilio-toxicity studies, using cilio-stimulatory and cilio-inhibitory test compounds.

The CBF of a total of 119 cells was recorded by the photometry system and the corresponding 119 areas were recorded by the high-speed camera system.

The means (± s.d.) of all control CBF values (between 10 and 30 min and after rinsing) determined by the photometry and high-speed digital imaging method were in good agreement: 8.2 ± 0.9 Hz (range 6.8–10 Hz; n = 40) and 7.9 ± 1.1 Hz (range 5.1–10 Hz; n = 40), respectively. The data obtained by the two methods are consistent with the range of CBF values reported for in-vitro conditions determined at room temperature, using the photometry method (Jorissen & Bessems 1995; Boek et al 1999; Jorissen et al 2000; Dimova et al 2003).

To assess the applicability of the high-speed digital imaging method for CBF measurements in a wider range of frequencies, and thus to validate its potential for cilio-toxicity studies, glycocholate (0.5%) and chlorocresol (0.005%) were used as cilio-stimulatory and cilio-inhibitory compounds, respectively. The absorption enhancer, glycocholate, and the lipophilic preservative, chlorocresol, have been previously used to assess the in-vitro ciliary reactivity of human nasal epithelial cell cultures, using the computerized microscope photometry method (Agu et al 1999; Dimova et al 2003). The CBF was measured between 10 and 30 min after exposure to the model compounds and after withdrawal of the xenobiotic exposure (after rinsing and subsequent incubation between 25 and 45 min with medium only).

After 10 to 30 min of exposure to medium only, the control CBF values determined by the photometry and high-speed digital imaging methods (n = 20) were 8.2 ± 1.0 Hz (range 6.8–10.0 Hz) and 7.8 ± 1.1 Hz (range 6.3–10.1 Hz), respectively (Figure 1). Although only 4% difference was observed between the mean control CBF values determined by the two systems, the paired Student's *t*-test showed statistically significant higher CBF when the photometry system was used.

The CBF values after 10 to 30 min exposure to chlorocresol (0.005%) (n = 20) determined by the photometry and high-speed digital imaging methods were 5.0 ± 0.9 Hz (range 3.4–7.0 Hz) and 5.1 ± 1.1 Hz (range 3.5–7.3 Hz), respectively (Figure 1). A similar cilio-inhibitory effect (of 38 and 35%) was measured by the two methods.

Similar CBF values were obtained with the photometry system and high-speed digital imaging method after exposure (10 to 30 min) to the cilio-stimulatory compound glycocholate (0.5%) (n = 19): 9.8 ± 1.0 Hz (range 7.5–11.8 Hz) for the photometry system and 9.7 ± 0.8 Hz (range 8.2–11.6 Hz) for the high-speed digital imaging method (Figure 1). The percentage increases compared to the corresponding control value were 20 and 24%, respectively.

The reversibility of the cilio-stimulatory and cilio-inhibitory effect of glycocholate and chlorocresol, respectively, after withdrawal of exposure was shown by the two methods used for CBF measurement (Figure 2). The mean CBF values obtained by the photometry and high-speed digital imaging methods were comparable in all experimental groups. A small (4%) but significantly lower CBF was determined with the high-speed digital imaging method compared with the photometry method in the chlorocresol pre-treated group.

The results obtained by the photometry and high-speed digital imaging methods, using perfusion system cultures, are in agreement with previously reported results concerning the reversible cilio-stimulatory effect of glycocholate

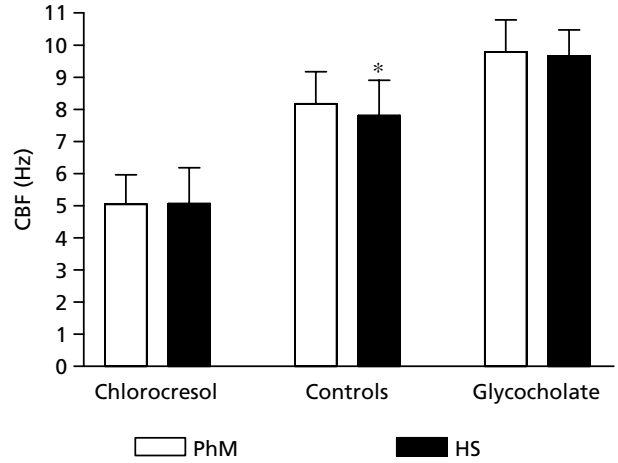

Figure 1 CBF of human nasal epithelial cells after 10 to 30 min exposure to medium (control), chlorocresol (0.005%) and glycocholate (0.5%) determined by the photometry (PhM) and high-speed digital imaging methods (HS). The results are expressed as mean ± s.d.; n = 19–20. *$P < 0.05$ vs the corresponding CBF value determined with the photometry method; paired Student's *t*-test.

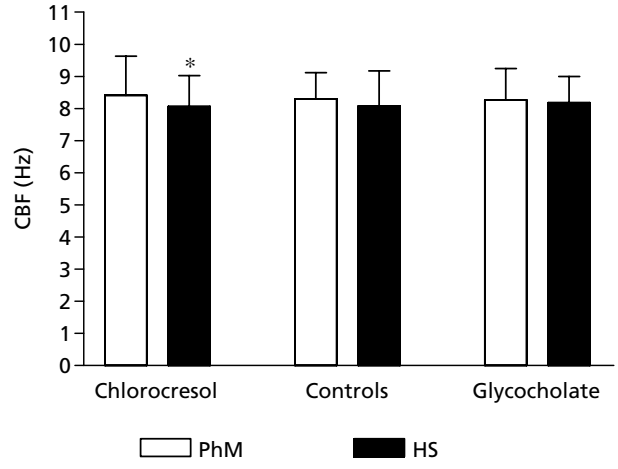

Figure 2 CBF of human nasal epithelial cells 25 to 45 min after xenobiotic withdrawal determined by the photometry (PhM) and high-speed digital imaging (HS) methods. The results are expressed as mean ± s.d.; n = 20. *$P < 0.05$ vs the corresponding CBF value determined with the photometry method; paired Student's *t*-test.

and reversible cilio-inhibitory effect of chlorocresol on the CBF of human nasal epithelial cells in monolayer and suspension cultures obtained with a Leitz MDV C2 photometry system (Agu et al 1999; Dimova et al 2003).

Although similar mean and s.d. values have been obtained with the two methods in all experimental groups, a significantly lower (by 4%) CBF was determined with the high-speed digital imaging method compared to the photometry in two out of six groups (Figures 1 and 2), which can probably be attributed to observer-related cell selection bias inherent to the photometry method. Using the photometry method, selection bias is difficult to avoid because the human eye is naturally drawn towards faster beating cells (Sisson et al 2003). The main limitations of the photometry method are: (i) the CBF of a single cell is recorded, (ii) the operator selects the cell to be analysed and (iii) the data acquisition is time-consuming (1 min for one cell). Most of the limitations of the photometry method can be overcome by the high-speed digital imaging method presented. The main advantages of the approach proposed are: (i) the CBF of an area with many ciliated cells is recorded, (ii) automated estimation of the CBF of all motile areas (pixel by pixel) of the recorded image, (iii) automated calculation of the mean CBF ± s.d. of all motile areas presented in the captured image and (iv) faster data acquisition (approximately 2 s for more than one cell), allowing a more complex experimental design.

A good linear correlation between the CBF values obtained with the photometry and high-speed digital imaging methods was observed in a wide range of frequencies (Figure 3; data from Figure 1). The agreement between CBF analysis methods was determined using the method suggested by Bland & Altman (1986). The Bland–Altman limits of agreement were calculated from the mean difference ± twice the standard deviation of the differences between the values obtained with the photometry and high-speed digital imaging methods. The Bland–Altman plot is presented in Figure 4.

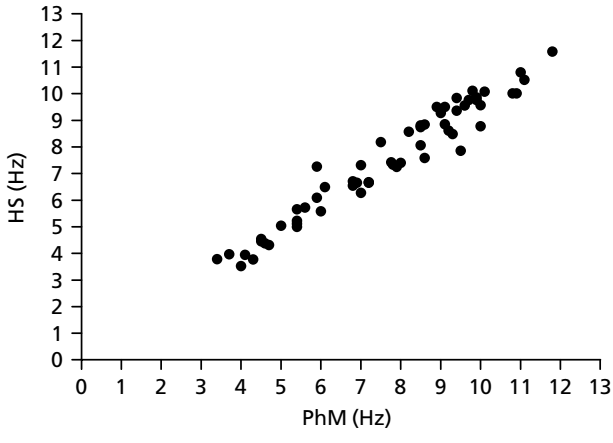

Figure 3 Correlation between the CBF values obtained with the photometry (PhM) and high-speed digital imaging (HS) methods after 10 to 30 min exposure of human nasal epithelial cells to medium (control), chlorocresol (0.005%) and glycocholate (0.5%).

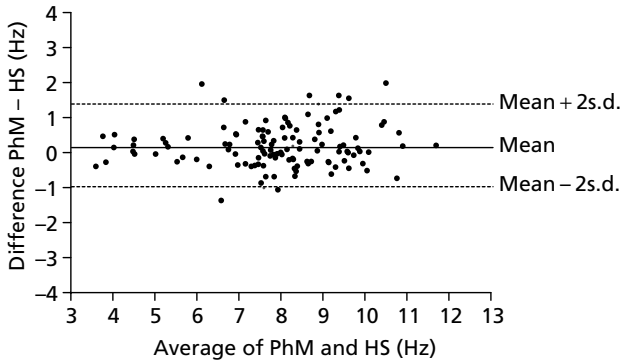

Figure 4 Bland–Altman plot of the high-speed digital imaging method (HS) compared with the photometry (PhM) method for CBF determination (24°C).

The mean difference between the photometry system and the high-speed camera method was 0.19 ± 0.6 Hz, and the Bland–Altman limits of agreement were from − 1.0 to 1.39 Hz. The Bland–Altman limits of agreement between the photometry and the high-speed digital imaging methods, determined in this experiment, were much lower compared to the reported limits of agreement between the photometry method and digital high-speed images (400 frames per second) from −2.75 to 5.15 Hz (Chilvers & O'Callaghan 2000), and digital video camera (85 FPS) from −3.89 to 3.39 Hz (Sisson et al 2003). Our method for whole field CBF analysis is largely analogous to the method previously published by Sisson et al (2003), but differs in several important factors, including a higher sampling frequency (500 FPS instead of 85 FPS), another approach for automatically discriminating between motile and non-motile regions (based on the standard deviation of the intensity variation over time in each pixel compared to the whole of the image) and a better spatial resolution.

The narrow limits of agreement between the photometry and the high-speed digital imaging methods show the reliability of the high-speed digital imaging method and the software used for CBF recording and calculations, and suggests that the results obtained by the two methods could be used interchangeably.

In conclusion, a reliable high-speed digital imaging and software system has been developed for in-vitro CBF measurements over a wide frequency range (3–12 Hz). The advantages of the system are: (i) fast data acquisition and calculation, (ii) whole-field automated CBF analysis and (iii) reduction in selection bias.

References

Agu, R. U., Jorissen, M., Willems, T., Van den Mooter, G., Kinget, R., Augustijns, P. (1999) Effects of pharmaceutical compounds on ciliary beating in human nasal epithelial cells: a comparative study of cell culture models. *Pharm. Res.* **16**: 1380–1385

Bland, J. M., Altman, D. G. (1986) Statistical methods for assessing agreement between two methods of clinical measurements. *Lancet* **I**: 307–310

Boek, W. M., Romeijn, S. G., Graamans, K., Verhoef, J. C., Merku, s F. W., Huizing, E. H. (1999) Validation of animal experiments on ciliary function in vitro. II. The influence of absorption enhancers, preservatives and physiologic saline. *Acta Otolaryngol*. **119**: 98–101

Chilvers, M. A., O'Callaghan, C. (2000) Analysis of ciliary beat pattern and beat frequency using digital high speed imaging: comparison with the photomultiplier and photodiode methods. *Thorax* **55**: 314–317

Deitmer, T. (1986) A method for standardizing cytologic sampling for the estimation of nasal ciliary activity. *Arch. Otorhinolaryngol*. **243**: 288–292

Dimova, S., Mugabowindekwe, R., Willems, T., Brewster, M. E., Noppe, M., Ludwig, A., Jorissen, M., Augustijns, P. (2003) Safety-assessment of 3-methoxyquercetin as an antirhinoviral compound for nasal application: effect on ciliary beat frequency. *Int. J. Pharm*. **263**: 95–103

Dimova, S., Vlaeminck, V., Brewster, M. E., Noppe, M., Jorissen, M., Augustijns, P. (2005) Stable ciliary activity in human nasal epithelial cells grown in a perfusion system. *Int. J. Pharm*. (in press)

Doran, S. A., Koss, R., Tran, C. H., Christopher, K. J., Gallin, W. J., Goldberg, J. I. (2004) Effect of serotonin on ciliary beating and intracellular calcium concentration in identified populations of embryonic ciliary cells. *J. Exp. Biol*. **207**: 1415–1429

Jorissen, M., Bessems, A. (1995) Normal ciliary beat frequency after ciliogenesis in nasal epithelial cells cultured sequentially as monolayer and in suspension. *Acta Otolaryngol*. **115**: 66–70

Jorissen, M., Willems, T., De Boeck, K. (2000) Diagnostic evaluation of mucociliary transport: from symptoms to coordinated ciliary activity after ciliogenesis in culture. *Am. J. Rhinol*. **14**: 345–352

Lale, A. M., Mason, J. D. T., Jones, N. S. (1998) Mucociliary transport and its assessment: a review. *Clin. Otolaryngol*. **23**: 388–396

Nasr, G., Schoevaert, D., Marano, F., Venant, A., Legrand, J. J. (1995) Progress in the measurement of ciliary beat frequency by automated image analysis: application to mammalian tracheal epithelium. *Anal. Cellular Pathol*. **9**: 165–177

Philips, P. P., McCaffrey, T. V., Kern, E. B. (1990) Measurement of human nasal ciliary motility using computerized microphotometry. *Otolaryngol. Head Neck Surg*. **103**: 420–426

Rautiainen, M., Matsune, S., Shima, S., Sakamoto, K., Hanamure, Y., Ohyama, M. (1992) Ciliary beat of cultured human respiratory cells studied with differential interference microscope and high speed video system. *Acta Otolaryngol*. **112**: 845–851

Rutland, J., Cole, P. J. (1980) Non-invasive sampling of nasal cilia for measurement of beat frequency and study of ultrastructure. *Lancet* **II**: 564–565

Sanderson, M. J. (2000) High-speed digital microscopy. *Methods* **21**: 325–334

Sisson, J. H., Stoner, J. A., Ammons, B. A., Wyatt, T. A. (2003) All-digital image capture and whole-field analysis of ciliary beat frequency. *J. Microscopy* **211**: 103–111