Compact device for assessment of microorganism motility

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We have designed and constructed a simple and reliable instrument, based on dynamic speckle interferometry, which allows the determination of the relative degree of motility for microorganisms. A measurement can be carried out in a few seconds and parasites have been used for test purposes. An *ad hoc* software was also developed showing easy operation characteristics. This instrument surpasses the purely qualitative methodology employed by human operators, that can be affected by great errors, fatigue, etc. Besides, it does not require using image formation systems (such as a microscope) and a frame grabber is not mandatory. © *2004 American Institute of Physics.* [DOI: 10.1063/1.1809266]

I. INTRODUCTION

Generally speaking, some biological essays (such as evaluation of drug action upon parasites or determination of sperm quality) require to determine degree of "activity" or motility of the involved microorganisms. It is clear that direct observation under the microscope allows, even when as a first approximation, one to decide if a given sample exhibits more or less activity than another. However, this technique suffers from certain obvious limitations: (i) the human observer can tire out, altering the final result of the experiment, (ii) different observers will not give, in general, the same result (especially if the samples to be compared are similar), and (iii) as the number of samples grows (three or more) it is more and more difficult for the observer to keep in mind a register of the observations which allows a reliable comparison between nonconsecutive samples. As an example, in veterinary parasitology, diverse authors have described an *in vitro* test based on larval paralysis which is affected by such subjectivity. Other alternative techniques include, for example, evaluation of motility in adult worms, making it necessary to sacrifice the infected animals. Presently it is possible to use charge coupled device (CCD) video cameras and image processing systems to obtain image sequences to be analyzed with adequate software. This approach is of particular interest when the individual behavior of the observed microorganisms must be considered, thus needing the tracking of a given number of them.[1,2] However, in some cases this may require relatively sophisticated equipment. On the other hand, many pharmacological and pharmacokinetic studies (in particular those concerned with drug action) only require information about the statistical behavior of the ensemble. In this point the speckle techniques are very useful, avoiding, for example, the need for image formation systems.

Recently, Pomarico *et al.*[3] described an experimental procedure based on *dynamic speckle interferometry*, also known as *biospeckle interferometry*,[4] for assessment of larvae motility. The approach was tested using the sheep parasite *Haemonchus contortus* (third-stage larvae, *L*3) incubated with three drugs: albendazole (ABZ), Ivermectin (IVM) and Levamisole (LVM) as an experimental model, showing good results.[3]

In this article we describe and test an apparatus for measuring the motility of microorganisms by means of the principle described in Ref. 3. It is portable and relatively lightweight and can be connected to any commercial frame grabber for image acquisition and processing, or can even been used with a quickcam. An *ad hoc* software has also been developed allowing easy operation and providing automatic data recording.

II. WORKING PRINCIPLE

A. Fundamentals of speckle

The measuring principle of the described apparatus relies on dynamic speckle interferometry. A speckle diagram arises when coherent light is reflected by a rough surface (in comparison with the wavelength of light). In this way, multiple wavelets, randomly dephased to each other, interfere at the observation plane giving rise to an (also) random granular figure. The same phenomenon can be observed when coherent light is passed trough a diffusive media, such as a ground glass. A detailed description about speckle diagrams can be found in Ref. 5. Additionally, if light is scattered by objects showing some kind of activity, the resulting speckle pattern exhibits a temporal variation in local intensities with the visual appearance of a boiling liquid. This is called a dynamic speckle pattern and can be caused by changes in refractive index, movement of the scatterers, or optical activity, among others. Some reported applications of this phenomenon include determination of parasite motility,[3] measuring the activity of seeds,[6] bruising of fruits,[7] etc.

B. Measurement algorithm

For determining the degree of activity of the phenomenon which gives rise to the dynamic speckle, the temporal

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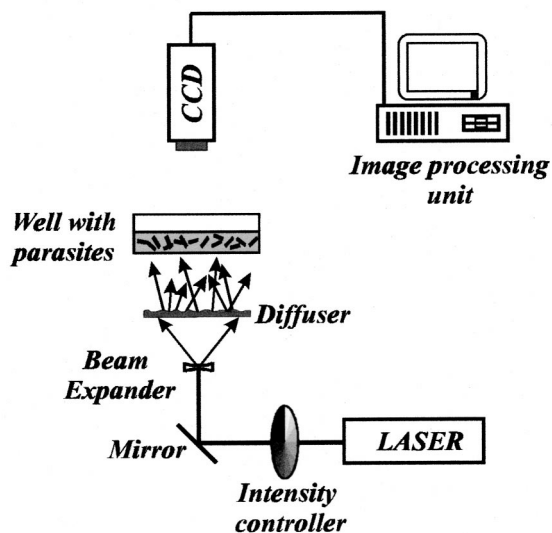
FIG. 1. Experimental setup used to record the dynamic speckle patterns due to parasite activity.

change in the speckle patterns needs to be assessed. To obtain a numerical value of this degree of change we used a generalized correlation algorithm based on the intensity difference between patterns. The algorithm can be applied to any phenomenon producing dynamic speckle patterns but we will refer to that concerned with microorganism motility. The experimental setup for this purpose is shown in Fig. 1 and consists basically of inserting the organisms which activity must be tested between a ground glass (which originates the speckle pattern) and the detection system (a CCD camera producing a gray levels image). The coherent illumination is provided by a diode laser. As stated above, it is necessary to quantify the temporal change in the speckle patterns. To this end, as a first step, a short movie of the dynamic speckle pattern is acquired at video rate (usually 30 frames/s) and a set of successive frames (typically 30) are stored. Depending on the frame grabber, the time interval between them can be chosen, but it is usually set to the normal value of $\tau = \frac{1}{30} s$. Given both, the intensity I (gray level) reaching the CCD detector at pixel (i,j) at time t and the intensity at the same pixel at time $t+\tau$, the required evaluation of temporal change can be obtained from

$$D(\tau) = \frac{1}{N} \sum_{ij} |I(i,j,t) - I(i,j,t+\tau)|, \quad (1)$$

which is a modified version of a temporal correlation.[3,8] N is a proper normalization factor and summation is done over the entire image. Accordingly with this last equation, very active speckle patterns, that is speckle patterns produced by organisms moving fast (and/or by high concentrations of organisms) will produce (for a given τ) significative intensity variations and thus $D(\tau)$ will be large. On the contrary, organisms moving slowly (and/or low concentrations of organisms) will produce small values of $D(\tau)$ (the speckle pattern remains almost the same). The highest motility (concentration) detectable depends on the speed of the camera, but, in general, the standard rate of 30 frames/s is enough. Even when computation of Eq. (1) does not require long process-

ing time for images as large as 512×512 pixels, image size can be substantially reduced without altering the final result. This is due to the enormous amount of points considered containing essentially the same information. The last statement relies on the fundamentals of speckle: when using free space propagation every single scattering element (in our case the moving microorganisms) contributes to the final intensity at each point being registered. Thus, the only requirement for the samples is to stay within the area illuminated by the laser, a condition which can be trivially fulfilled. However, it must be noticed that individual tracking is lost with this approach.

III. CONSTRUCTION DETAILS

Figure 2(a) shows a schema of the main components of the apparatus manufactured accordingly with the experimental setup of Fig. 1, while Fig. 2(b) is a photograph of the actual device. It consists basically of three main modules. In the first module (the emission or illumination module), a 3 mW laser diode (LD) operating at $\lambda = 670$ nm provides coherent illumination. Power is provided through a wall transformer and the intensity is controlled by means of a linear polarizer next to the laser. A ground glass provides a fully developed speckle pattern.[5]

In the second module, the organisms to be tested are placed in 0.3 cm^3 cylindrical wells of about 5 mm diameter. These are standard laboratory wells mounted together in a 1×8 strip. The space between cylinders is used to keep the strip in place by means of two little metal spheres which are pressed towards the center by two springs. This simplifies the positioning and identification of the wells, especially when many measurements are to be carried out. A small aperture is inserted at the top of this module to provide control of the mean speckle size. This is a very important point since speckle grains must be well resolved by the CCD detector; otherwise the system will be insensible to intensity changes. Speckle grains are considered to be well resolved by the CCD array if they extend over several pixels (10 can be considered a good guiding rule) on the detector. Figure 2(c) shows a close-up picture of this module.

The third module contains a CCD camera which detects the speckle patterns in a free space configuration (no image formation is required). The camera is placed about 25 cm away from the wells. A tube connects the second module with the camera to prevent incoming of stray light. It also serves, together with the pinhole at the top of module 2, to define the mean speckle size (δ) on the CCD, which for free space configuration is given by the well known relation[6] $\delta = 1.22\lambda D/d$, d being the pinhole diameter and D the distance between the pinhole and the CCD sensor plane.

The final processing is carried out in a PC. A frame grabber is not mandatory, and a quickcam can be used for speckle detection, provided that it has the required speed.

The whole assembly stands on a heavy iron base with three feet to provide the required stability. Total height from base to top of the camera is about 45 cm.
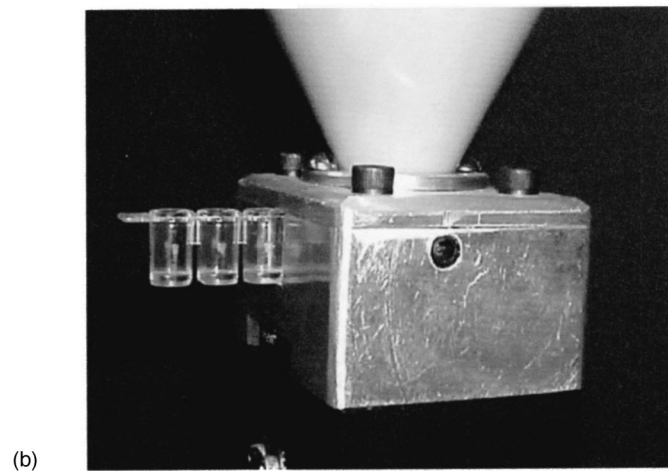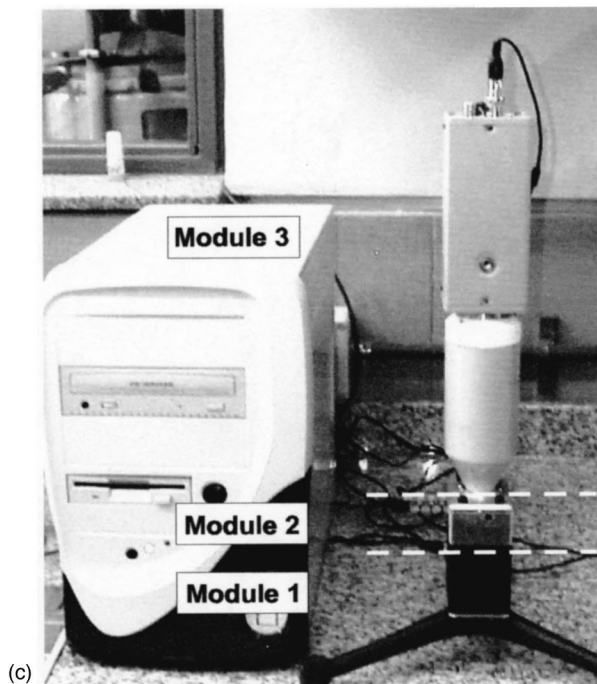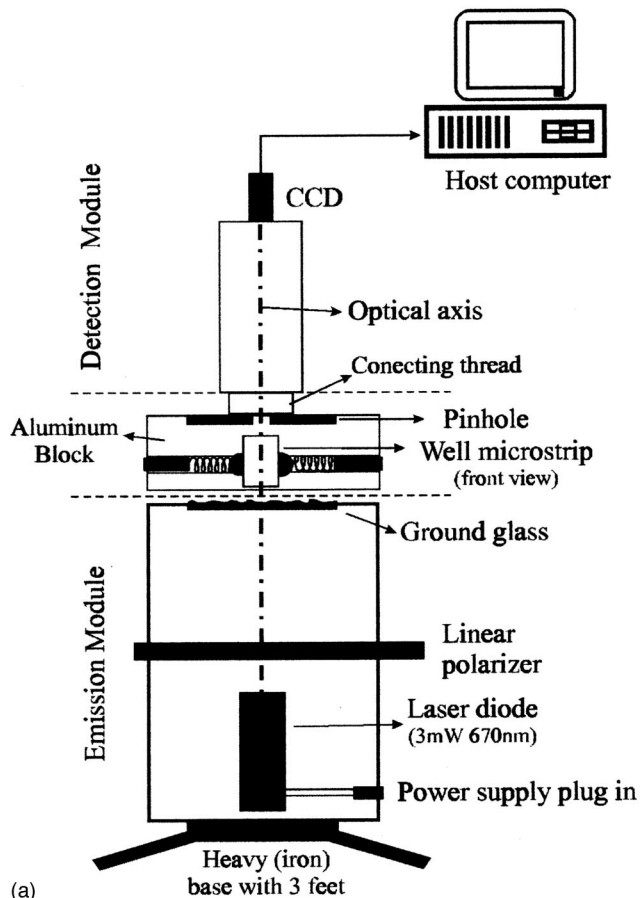
FIG. 2. (a) Schematic representation of the three main modules of the actual apparatus (not to scale). (b) Photograph of the manufactured device. The three modules described in Sec. III are identified. (c) A detailed picture of module 2 showing the wells where parasites are placed. Wells are 5 mm in diameter and 14 mm in height.

IV. SOFTWARE

Handling and processing of the images needs, depending on the laboratory equipment, some tedious operations which can be also time consuming. A typical motility measurement goes through three basic steps: (1) acquisition of a movie (lasting about 1 s), (2) cutting the movie in individual frames, and (3) evaluation accordingly with Eq. (1). In particular, when using a multipurpose commercial soft-

ware, obtaining a frame by frame sequence of the speckle diagrams originated by the process being studied requires (for each sample) some minutes. As is always the case with biological samples, many replications of each one are needed, making the total time for separating the frames quite large. Moreover, many commercially available software packages do not support the operation of frame by frame downloading.

With that in mind, a software was designed for simplifying the entire process and including some practical options for analysis and recording of data.

The application, called *Bichos* (a spanish word for bugs) runs under standard operating system. Additionally, some digital video acquisition device is needed. It can be an acquisition device having a digital output (such as a quickcam) or a video card for digitizing analog inputs coming from standard video cameras. In any case, the acquisition device (or devices if more than one) is (are) automatically detected and the program starts showing a live image. The main menu has three components:

(1) the *Device* menu, which allows to select the desired acquisition device. Once a device is selected it will remain as the default option until a new one is chosen. Selection of a given device allows the user to access the corresponding driver for options modification.

(2) The *Configuration* menu consists of two parts. The first one is related to the analysis options. With this menu it is possible to choose the image size. If the selected image size is greater than that defined in the acquisition options of the selected device, the size defined for acquisition will be used. The time step between images can also be chosen to be considered for processing as multiple integers of the frame rate. For example (for a movie lasting 1 s), if images are acquired at 30 frames per second, the selected time step can be $\frac{1}{30}s, \frac{2}{30}s, \ldots . 1s$. This is very useful if slow processes are being studied, since consecutive frames may not differ in a detectable way. Moreover, the number of image pairs considered (compatible with the time step selection) can be varied for averaging purposes. Clearly if, for example, the time step is set to $\frac{15}{30}s$ no more than two image pairs can be defined. This is automatically checked.

The second part of this menu gives the operator the option of recording or not the temporary data being produced during processing. Thus, one can optionally retain the movie (as AVI file), or the RAW files, etc. As default, the numerical results for the motility are kept in a directory named after the date and time of measurement.

(3) The *Details* menu allows one to access a frame by frame sequence of the video captured. By selecting a given image (a speckle pattern) a histogram is displayed together with its mean intensity, $\langle I \rangle$ and contrast, C defined as $C = \sigma/\langle I \rangle$, σ being the standard deviation of the intensity. These options are not useful for operators not familiar with speckle fundamentals, but allows a rapid evaluation of the goodness of the images being acquired. For example, images presenting too many saturation points are bad ones, since these points are insensible to moderate intensity variations.

After all options have been selected, the *Analyze* button initiates the process accordingly with the algorithm described above.

For large image sizes (512×512 pixels) and small time steps $\left(\frac{1}{30}s\right)$ the whole task of acquiring the movie, cutting the images and processing them lasts about 5 s. Numerical results are ordered after appearance in a column at the right of the screen.
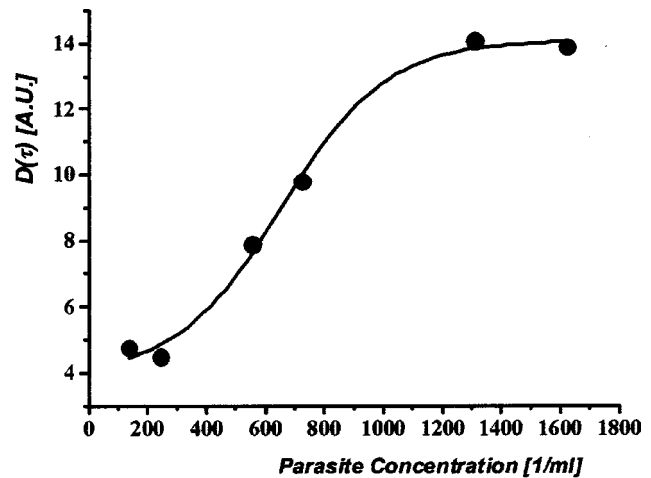

FIG. 3. Representation of the difference function, $D(\tau)$, as a function of the $L3$ stage larvae concentration. A clear sigmoidal plot ($R^2 = 0.995$) can be observed showing an approximate linear behavior for concentrations between 400 and 1000 mℓ^{-1}. For this experiment, larvae were not exposed to any drug.

V. RESULTS AND DISCUSSION

We have constructed a device that, applying the principles of dynamic speckle, allows the evaluation of microorganism motility. The main advantages of the reported technique are its objectivity, sensitivity, and fast recording and processing of the data.

Before any quantification of motility can be done it is important to investigate the influence of other parameters upon the final result. For example, when dealing with microorganisms, their concentration can change the numerical value of the correlation function $D(\tau)$, defined in Eq. (1), since every individual organism contributes to the whole activity of the ensemble. Also the dynamic range must be evaluated, due to the fact that for a given sampling time interval very low parasite concentrations do not produce any measurable activity of the speckle pattern and too high concentrations lead to a saturation of the measurement due to undersampling effects.

Results corresponding to the activity of different concentrations of $L3$ are shown in Fig. 3. The data adjust very well to a sigmoidal curve, reflecting the above statement, and is linear for concentrations between 400 and 1000 mℓ^{-1}. For this experiment the time interval between images was set to $\tau = \frac{1}{30}s$. This was checked by varying τ up to values as great as 0.1 s, where the obtained values of $D(\tau)$ began to fluctuate, thus indicating correlation loss. The plotted points are the result of averaging four values of $D(\tau)$ resulting from pairs of consecutive speckle images within a 1 s movie.

Among the most important applications of our device is the investigation of the effect of different drugs on microorganisms, since this directly affects their motility. Thus, we tested the equipment by investigating the effect of drugs on $L3$ stage larvae of *Haemonchus contortus* as a function of time. Results are shown in Fig. 4 and LVM (Sigma-Aldrich, Steinheim, Germany) was used as a typical drug for controlling this type of parasite. A concentration of 2 μg mℓ^{-1}
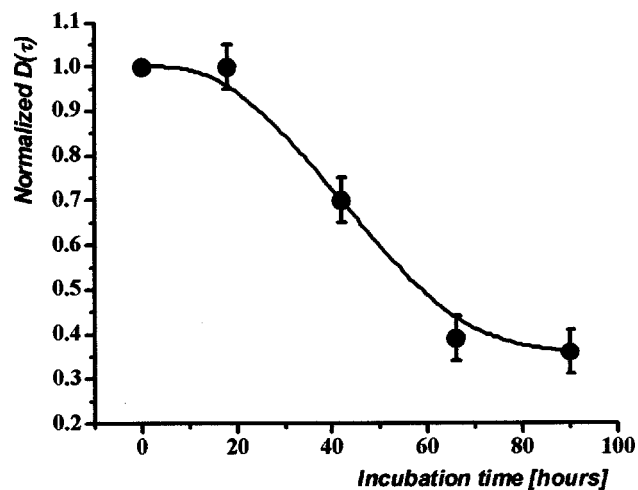
FIG. 4. Normalized time evolution of LVM activity. Please notice that the line connecting the experimental points does not represent any fitting, but is just one provided by the spline method.

(which is a pharmacologically relevant) was used, obtained from previously reported studies.

It should be noticed, that the curve in Fig. 4 is normalized to the motility value just before drug action starts, irrespective of parasite concentration provided that it lies in the linear region shown in Fig. 3.

As a conclusion, the proposed device can be successfully applied to quantification of activity of speckle fields, thus allowing assertion of objective numerical values to the phenomena being monitored with this principle.

ACKNOWLEDGMENTS

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