Photon Counts Statistics in Leukocyte Cell Dynamics

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Abstract. In the present experiment ultra-weak photon emission/ chemiluminescence from isolated neutrophils was recorded. It is associated with the production of reactive oxygen species (ROS) in the “respiratory burst” process which can be activated by PMA (Phorbol 12-Myristate 13-Acetate). Commonly, the reaction is demonstrated utilizing the enhancer luminol. However, with the use of highly sensitive photomultiplier equipment it is also recorded without enhancer. In that case, it can be hypothesized that photon count statistics may assist in understanding the underlying metabolic activity and cooperation of these cells. To study this hypothesis leukocytes were stimulated with PMA and increased photon signals were recorded in the quasi stable period utilizing Fano factor analysis at different window sizes. The Fano factor is defined by the variance over the mean of the number of photon within the observation time. The analysis demonstrated that the Fano factor of true signal and not of the surrogate signals obtained by random shuffling increases when the window size increased. It is concluded that photon count statistics, in particular Fano factor analysis, provides information regarding leukocyte interactions. It opens the perspective to utilize this analytical procedure in (in vivo) inflammation research. However, this needs further validation.

1. Introduction

Infections cause currently more than 20 percent of all human death in the world. Like all other animal organisms, the human survives this world with hostile and rapidly evolving pathogens by utilizing mechanisms to resist infection by pathogens. These defences are of two kinds: innate immune responses and adaptive immune responses. During the first critical hours and days of exposure to a new pathogen, humans rely on the innate immune system. The second, the adaptive immune system, which remembers previous encounters with specific pathogens, is too slow after a first exposure to a new pathogen. It can take a week before specific clones of B and T cells are activated and expanded, whereas a single bacterium with a doubling time of one hour can produce already a 20 million progeny infection in a single day. The innate immune system relies on the recognition of particular types of molecules that are common to many pathogens but are absent in the host. These pathogen-associated immune-stimulants stimulate two types of innate immune responses – inflammatory responses and phagocytosis by cells such as neutrophils and macrophages. The recognition of the microbial invader is usually quickly followed by its engulfment by a phagocytic cell. In vertebrates, macrophages reside in the tissues throughout the body and are especially abundant in areas where infections are likely to arise, including the lungs and gut. They are also present in large numbers in...
connective tissues, the liver and spleen. They patrol the tissues of the body and are among the first cells to encounter invading microbes. The second major family of phagocytic cells, the neutrophils, are not present in tissue of healthy subjects but are abundant in blood. These cells are rapidly recruited to tissue sites of infection [1].

Once the pathogen has been phagocytised, the macrophage or neutrophil kills the pathogen utilizing different strategies. The phagosome is acidified and fuses with lysosomes, which contain lysozyme and acid hydrolases that can degrade bacterial cell walls and proteins. In addition, the phagocyte assemble a special electron transport system complex (NADPH-oxidase) on the phagosomal membrane that catalyzes the production of a series of highly toxic oxygen-derived compounds, including superoxide anion ($O_2^-$), whereas subsequent reactions lead to the formation of other toxic agents including hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl) and possibly hydroxylradical (OH·) and singlet oxygen ($^1O_2$). The production of these toxic compounds is accompanied by a transient increase in oxygen consumption by the cells, called the respiratory burst. The respiratory burst [2] is intrinsically associated with low-level biochemiluminescence or ultra-weak photon emission. The production of ultra-weak photon emission by phagocytosing (polymorphonuclear) leukocytes correlates with their metabolic activation [3].

A fascinating aspect of the neutrophil is the rhythmicity in the process of metabolic activation. In fact, almost every organism examined displays some form of rhythmic activity. Moreover, within a single organism almost every major organ system can, under appropriate circumstances, generate sustained oscillations. The periods of oscillation can range from a fraction of a second to several hours. At cell level, the neutrophilic leukocyte is a well-studied example of this type of regulation, in particular the amplitude and frequency modulation of intracellular NADPH concentrations. With respect to the cellular spatial-temporal organization, it has been observed, that NAD(P)H oscillations are in-phase with oxidative oscillations and that superoxide production follows NAD(P)H amplitude and frequency changes. It has been argued that associated kinases and phosphatases play a role in NADPH oxidase phosphorylation and in decoding these fluctuations into series of superoxide bursts [4] and periodic cytolytic events [5].

Although these studies are limited to the amplitude and frequency modulation of cellular metabolic oscillations, there is also some indication for the existence of intercellular communication. Thus, neutrophils appear to detect time-varying chemical fields displaying this same period [6]. Other research data [2] suggest a role of the neutrophil’s ultra-weak radiation in stimulating, in a non-chemical, non-electrical way, the metabolic activity of other neutrophils which are spatially (but not optically) separated. These intercellular communication aspects suggest that sustained oscillations in ultra-weak photon emission may be even displayed within populations of leukocytes cells.

Collective regulation has attracted the attention of researchers from the 1970’s and onwards for its association with the statistical properties of the ultra-weak photon emission. However, it is difficult to develop reliable procedures for the use of ultra-weak photon emission in the detection of cooperative phenomena in cell populations. Oscillations in intensity have been demonstrated in a few cases. A distinct type of oscillations in ultra-weak luminescence with a period of about 1 h was for instance observed in cell populations using Nitellopsis obtusa cells [7]. In another study, long-period oscillations with a period of a multiplex of 3 min have been measured in the photon emission of a culture of dark-adapted Acetabularia cells utilizing Fourier analysis [8].

In the present study, we document on the possibility to apply novel photon count distribution analytical methods in cell population studies, in particular for neutrophil cell population and their respiratory burst associated photon emission. We have focussed on the detection of ROS related single photon emission without enhancers. This is a novel development since almost all studies record photon emission that has produced by chemical
substituents like luminol or lucigenin which react upon reactive oxygen species (ROS) with the production of photons. Studies on the detection of photon emission without enhancers are very limited, mostly due to the sensitivity of the detection equipment. However, just this latter is of increasing importance for obtaining information from time resolution studies on the photon signal directly associated with metabolic processes. The use of high-sensitivity, low-noise photomultiplier equipment in combination with novel photon count statistics permit documentation of photon signals within the concept that photons in a biological system are part of an integrated organization of biochemical reactions. Such studies have indicated that photon count distribution tends to a super-Poisson distribution which implies bunching of emitted photons indicating clustering of excitation processes [9-11]. The opposite phenomenon, anti-bunching of photons indicated by a sub-Poisson distribution of photon counts, may be initiated under conditions of excitation feedback. It has been reported in early laser studies but has not reported for living systems [12].

Super, normal or the sub-Poisson nature of a photo count distribution is easily ascertained by the value of its Fano factor [13]. The Fano factor is the ratio of the variance of the number of photons detected in many repeated measurements with a counting time window of duration \( T \) to the mean number of photons in the same set of measurements. It is written as \( F(T) = \frac{\text{Variance} [N_i(T)]}{\text{mean} [N_i(T)]} \) where \( N_i(T) \) is the number of events in the \( i^{\text{th}} \) time window of length \( T \). Within this context, its value is greater than one for super-Poisson, is equal to one for Poisson distributions and is less than one for sub-Poisson distributions. The Fano factor may be dependent on window duration and estimation of Fano factor values for increasing window sizes then results in a Fano factor time curve. In some biological systems, the \( F(T) \) shows an increase because rare clusters of high and low photon counts are more apt to be found as more and more data are collected. Such clustering has been considered characteristic of a fractal process [14]. Fano factor time curve was utilised before to study the statistical properties of action potentials [15-18], ultra-weak photon emission data from micro-organisms and human subjects [11, 19-21].

We considered it important to utilize Fano factor analysis in the study of populations of cells when photon emission is a rare event. Thus, when populations of thousands or millions of cells show a photon emission of less than hundred photons per second, photon emission from individual cells is considered very rare. The emission from an entire population of non-interacting cells is expected to result in Poisson distribution of photon counts. Any deviation towards super-Poisson suggests cell interaction; the signal contains information within the sequence of photon counts events about the population as collective phenomenon.

The presented experiments document the results of \( F(T) \) analysis of ultra-low level photon emission measured from isolated neutrophil leukocytes, after stimulation of the “respiratory burst” process by Phorbol 12-Myristate 13-Acetate (PMA). The chemiluminescence emitted during this reaction is recorded with the use of highly sensitive photomultiplier equipment without chemical enhancer. Photon count statistics and \( F(T) \) analysis are reported and suggest that neutrophils as a population, display an integrated metabolic organization.

2. Material and Methods

2.1. Neutrophils isolation

Neutrophils were isolated from freshly drawn whole blood from pigs that was collected and heparinised at the moment the pigs were aborted. At four occasions, a quantity of 500 millilitre (ml) was collected, divided in three portions and stored at 4°C for use at three consecutive days after collection meaning that we had twelve experimental days. During each of the twelve experimental days, neutrophils were isolated in three independent sessions. In each session, 50 ml blood was utilized. In principle, isolation was performed following the method described earlier by Shen et al (1994) [2]. In short, neutrophils were isolated from 50
ml heparinized whole blood after dextran (Sigma-Aldrich, St. Louis, MO) sedimentation of erythrocytes for 45 minutes. After isolation, neutrophils were washed by centrifugation and resuspension in Hanks’ Balanced Salt Solutions (HBSS) (Sigma-Aldrich St. Louis, MO) in three cycles. Cells were finally resuspended in fresh HBSS and counted before use. Neutrophil viability, assessed by the ability of the cells to exclude trypan blue (Sigma-Aldrich St. Louis, MO), was always more than 90%.

2.2. Experimental protocol
The reported experiments on photon emission were carried out with neutrophil cells incubated at 25°C. Before initiating photon emission, 10 ml of neutrophil suspension in HBSS (approximately 1.5x10⁷ cells) was placed in a Petri dish of 5 cm diameter and equilibrated at 25°C for 15 min in the dark chamber of the photomultiplier equipment. After this temperature equilibration, 10µg phorbol myristate acetate (PMA) (Sigma-Aldrich St. Louis, MO) was added to the Petri dish to induce the respiratory burst. There was a delay of 7 sec between adding the PMA and the first moment of measurement. This was due to the fact that PMA was added to the cells, closing the measuring chamber and starting the measurement. The photon emission was recorded during 2400 s. In thirty six experiments, photon emission was measured without using enhancer substances. In twelve experiments luminol was present at a concentration of 1µg/ml during the incubation and photon emission measurement. This resulted in a total of thirty six curves without and 12 curves with luminol for data analysis (see below).

2.3. Photon emission measurements
Photon emission was recorded with a vertically placed, cooled (25°C) photomultiplier tube (H-R550, Hamamatsu Photonics Co. Ltd, Hamamatsu, Japan), that was placed in a vertical position on the top of the incubation chamber. The opening window of the photomultiplier was 46 mm. The distance between the sample and photomultiplier tube was 10 cm. The temperature of the sample (Petri dish) holder was controlled at 25°C by a Peltier-element. Recording was performed with consecutive 50 millisecond (ms) periods. Technical aspects and data acquisition were computer controlled.

2.4. Data analysis
The photon emission signal was screened for artefacts. The sources of artefacts are primarily cosmic ray-induced photoelectron clusters or micro discharges occurring in the PMT’s. These artefacts are clearly detectable. For correction, values larger than 15 times the standard deviation in the entire data row were replaced by mean values. Because the removal of artefacts influences the standard deviation of the overall data set this procedure was repeated until no artefacts were found. Two type of data analysis were made. First, we looked at the intensities in time to estimate the time course of photon emission produced by the cells during their respiratory burst. Subsequently, we estimated the period that the induced signal was quasi-stable. The term quasi-stable period has been used because the overall signal in this period shows fluctuations without an overall upward or downward trend. For this purpose, time fragments were selected which on one hand have no significant upward or downward trend and, on the other hand are sufficiently long to estimate the Fano factor dependency on window times up to 50 s (see below). This resulted in the selection of 3-4 non-overlapping fragments of 5 min for each emission curve. It ultimately resulted in a set of 120 data rows for the Fano factor analysis without enhancer and 40 data rows with luminol.

2.5. Fano factor analysis
The value of F(T) represents the Poisson nature of a photo count distribution [14]. Its value is greater than one for super-Poisson distributions, is equal to one for Poisson distributions and is
less than one for sub-Poisson distributions. Since background, due to electronic noise also
demonstrates a deviation from a Poisson value of unity, one has the option to correct for
background assuming that the sources of photon counts originating from signal and
background are independent for measurements of all varieties of time windows. In the case of
a low signal compared to noise, however, such correction decreases the reliability of the
estimations. On the other hand, such correction is not necessary when one is only interested in
the relationship between Fano factor value and time window used for analyzing the photon
data. In the present analysis, we calculated this relationship both for background signals and
neutrophil population signals (which also included the background).
Whether the Fano factor time curve truly reflects long-range correlations of events was tested
by constructing surrogate data sets based on the same data but after randomization of these
data. These surrogate data sets were obtained 10 times of shuffling randomly the values in the
primary observed signal.

2.6. Statistics
Statistica 6.0 was used for basic statistical analyses of photon count data.

3. Results

3.1. Kinetics of PMA-induced photon emission
The PMA-induced photon emission during the respiratory burst of the leukocyte cell
population is illustrated in Figure 1 by a representative example.

![Figure 1](image)

**Figure 1.** Spontaneous photon emission of leukocytes during respiratory burst after
stimulation with PMA (without enhancer).

The data in this representative curve show initially a value of about 20 counts per sec (cps),
which is followed by an increase in photon emission starting 500 sec after the addition of
PMA. In the initial period we have not detected any difference between the photon signal of
cells and background. The initial (0-500 sec) values represent background from electronic
noise; it is similar for backgrounds measured with shutter open and shutter closed. In the
experimental period 36 backgrounds were determined; the average background values (±std)
was 18.3 ± 0.9 cps. The increase starting after 500 sec was highly typical and continuing up to
1000 sec. From 1000 sec on photon emission only slowly decreased and allowed the selection
of consecutive 300 s time periods with quasi-stable photon emission in the period of 1000-2400s for photon count statistical analysis and F(T) estimations.

These kinetics suggest that after PMA addition there is a 500 sec initial lag in ROS production, followed by an increase and then a period of relatively constant production. To be certain about the initial lag in ROS production, similar incubations were performed in which the enhancer luminol was present. A representative example of the kinetics of photon emission in the presence of luminol is presented in Figure 2. Data suggest that a similar lag period is observed when the enhancer luminol was used, suggesting that the initiation of ROS production requires such period of time.

![Figure 2](image)

**Figure 2.** Luminol enhanced photon emission of leukocytes during respiratory burst after stimulation with PMA. The black curve represents moving average.

The presented example with luminol, demonstrates that under these conditions the actual photon signal (corrected for background) in the quasi stable period is about 300 times higher as compared with the emission without luminol.

3.2. F(T) of PMA-induced photon emission

Increased photon signals recorded in the quasi stable period were used to calculate the behaviour of the Fano factor F(T), regarding different window sizes. The window sizes ranged between 50 ms and 50 sec measurement period. The longer periods include combinations of 50 ms measurement periods. The analysis is limited to the 50 sec maximum because reliability strongly decreases with less than 6 windows. This procedure was executed both for leukocyte data and background. Moreover, to demonstrate the presence of sequence related phenomena, the Fano factor of true signal was compared with surrogate signals obtained by random shuffling the original data.

First, the F(T) in background data was estimated utilizing thirty six background datasets. The Fano factor data at different windows were calculated for each individual dataset and then these individual F(T) curves were averaged. The mean F(T) curve is shown in Figure 3. The data show that the Fano factor is about 1.25 and it is not increasing with larger window sizes. It has the same flat curve and average value as permuted background data. The super Poisson nature is a common feature for photomultiplier tubes and has been ascribed previously [20].

Similar analyses were made for cell populations of leukocytes utilizing 120 experimental data sets. It was demonstrated that the Fano factor increased definitively with window time.
The F(T) curve representing the average of the 120 curves is presented in Figure 4. In contrast, the F(T) in permuted series is not increasing. The increase in the F(T) curve of real signals occurs because rare clusters of high and low photon counts are more apt to be found as more and more data are collected; a clustering which has been considered characteristic of a fractal process.

Figure 3. Fano factor curve F(T) averaged over 36 background measurements (open symbols represent average over background curves; closed symbols represent average over background curves after shuffling the data).

Figure 4. Represents the average F(T) over the 120 data sets of leukocyte cell populations (open symbols represent average over measured data; closed symbols represent average over shuffled data).

It must be emphasized however, that these curves were derived from data including the actually photons from PMA-stimulated photon emission and electronic background. Within the entire set of data, subsets are present with high and with low photon signal compared to electronic counts. It can be hypothesized that high and low photon data sets are significantly different with respect to their F(T) curve. In Figure 5, a comparison is made between F(T) of all neutrophil data sets with and without shuffling and the F(T) curves of the 30 data sets with highest and 30 data sets with lowest intensity signal. The data confirm the effect of signal
intensity on the shape of $F(T)$ curve. The higher the intensity, the higher the increase of the $F(T)$ curve, demonstrating the influence of background values with its flat $F(T)$ curve (Figure 3).

![Figure 5](image)

**Figure 5.** $F(T)$ curve of all leukocyte data sets (with and without shuffling), and $F(T)$ curves of data sets from 30 highest and 30 lowest photon emission intensity.

3.3. $F(T)$ curves of low-intensity artificial light

For comparison the $F(T)$ curve of artificial light of similar intensity as high levels of PMA-induced photon signals was estimated. For this purpose, light emission from a light-emitting diode (LED) (Conrad Electronic, Type TLLG 5400, green) was measured and analyzed following the same procedure. With the use of grey filters (Jenaer Glaswerk Schott & Gen, Mainz, Germany) the intensity was reduced to 30 cps (including background) without compromising the stability of the signal. A total of 50 data sets as collected. The average $F(T)$ curve, presented in Figure 6, illustrates that the Fano factor over the entire range of windows was about 1.25, similar to background. The Fano factor of the surrogate, shuffled data sets has the same value.

![Figure 6](image)

**Figure 6.** Fano factor curve $F(T)$ averaged over 50 LED data sets (open symbols represent average over LED curves; closed symbols represent average over LED curves after shuffling the data).
These data are another argument that the photon emission signal of the neutrophil cell population, in contrast to artificial light or background, contains some decipherable information in the form of an increasingly long-range correlation with longer window times.

3.4. F(T) curves of PMA-induced luminol dependent photon emission
As indicated before, a series of 40 data sets on PMA-induced photon emission in the presence of luminol have been collected. A representative curve of this collection is shown in Figure 7. We intended to make the same comparison between F(T) curves of luminol-dependent photon emission data sets with high and low intensities.

Figure 7 illustrates F(T) curve of all luminol measurements (N=40), and F(T) curves of measurements from 20 highest and 20 lowest intensity data sets. Data demonstrate that the slope of F(T) curve also depends on intensity of the signal: the higher the intensity the higher the increase with window size. In shuffled data this increase totally disappeared.

4. Discussion
Production of ROS in health maintenance is of major relevance with regard to the capacity of phagocytic cells, which are a major producer of superoxide O$_2^-$ in response to invading microorganisms. In phagocytic cells, a burst of O$_2^-$ important for killing microorganisms is produced by a multicomponent cell membrane-bound NAD(P) upon receiving specific membrane signals.

The utilization of spontaneous ultra-weak photon emission to register ROS production in cells and tissues has been extensively described. For an overview the reader is referred to extensive classic and recent reviews [22,23]. The detection of photon energy from tissues or cells is a rare event. A spontaneous photon emission of approximately 10 photons / s was registered. It must be emphasized that the actual number of photons emitted needs a variety of factors to be included. Among them are the spectral sensitivity of the photomultiplier, the instrumental characteristics like the size and shape of the sample holder and photomultiplier. Other factors relate to the emitting biological material, namely its homogeneity within the Petri dish and the number of cells. The latter factors influence the absorption by cells other than emitter cells. Apparently, the actual number of photons registered represents a percentage of photons leaving the cell. In tissues, composed of multilayers of cells one can imagine a
large fraction of emitted photons may be absorbed. In case of a cell monolayer as in the present experimental conditions with neutrophils, this absorption is relatively small. The spectral sensitivity is estimated at approximately 20%. We have checked microscopically the spatial distribution of cells as well as the image of photon emission from the neutrophil cultures using a highly sensitive CCD camera. It was demonstrated that the cells tend to cluster and adhere to each other, covering the Petri dish surface for about 35%. From the distance between Petri dish and the opening of the photomultiplier we estimated that 1.2% of the photons reach the tube. The entire set of these conditional circumstances led to the conclusion that leukocytes emit approximately 1 photons per cell during a time period of 20 min to 1 h. In this respect, the emission indeed is a rare event. Therefore, the deviation from a Poisson distribution is a good indicator for a form of communication between processes involved in cells producing photon emission.

The fluctuation in observed values of rare events is represented by a Poisson distribution. Fano factor [14], is a good indicator of the deviation of observed values from a Poisson distribution; in case this ratio is higher than one, this is called super-Poisson. This ratio has been previously utilized for characterizing spontaneous photon emission of bacteria [11], Dictyoselium [19] and human hands [20,21]. In the previous studies it was also described that the Fano factor was dependent on time windows. In these studies, like in the present ones, it was checked that such increase completely disappeared in surrogate series obtained by randomly shuffling or permutation of the 50 ms photon data. The common explanation for the increase in variance is that in longer window lengths the more rare clustering of photon signals is revealed.

The data can be interpreted as argument for the occurrence of fluctuations of some sort of multi-frequency nature. Studies on periodic changes in ultra weak photon emission are hardly performed, except in a few cases. Highly regular fluctuations have been described for soybean roots. In those experiments, photon emission as well as the surface potential have been chosen because both can be measured without damaging the specimen [25]. In the time course, photon emission intensity changes almost synchronously with variation of the surface potential. Other example, with less regular periodic oscillations in ultra-weak luminescence are the cell populations of Nitellopsis obtusa cells with a major period of about 1 h [7], and dark-adapted Acetabularia cell populations with a period of a multiplex of 3 min [8].

In most cases, however, the fluctuations in photon emission are highly irregular and questions have been raised about its random or otherwise chaotic (for instance fractal) nature. In the present experiments, the F(T) curve showing increased Fano factor with increasing window size points to the latter explanation, i.e., fractality. This means that cells interact. This interaction however, needs further clarification.

The most attractive model for cell interaction and intercellular dynamics has been the glycolytic pathway of yeast cells in culture. It appeared that under certain aerobic and anaerobic growth conditions, the concentrations of metabolites of the glycolytic pathway oscillate [25,26]. For the oscillations to be observable, they had to be synchronous in the billions of cells in the cuvette. Indeed, mixing of cell populations that oscillated 180 degrees out of phase, caused a transient disappearance of the oscillations, evidencing some sort of active synchronization. It also implies that the dynamic phenomenon of the oscillation propagates within and between cells. These results were in favor of a concept that metabolic processes are able, in principle, to oscillate in a collective manner within intact individual cells as well as within cell populations.

In summary, the data led to the conclusion that Fano factor analysis offers a perspective to estimate frequency modulation of intercellular metabolite interactions. The encoding of this signal information is of interest for research aimed at defining states of (chronic) inflammation, like in rheumatoid arthritis. However, this needs further validation in animal models or humans.
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