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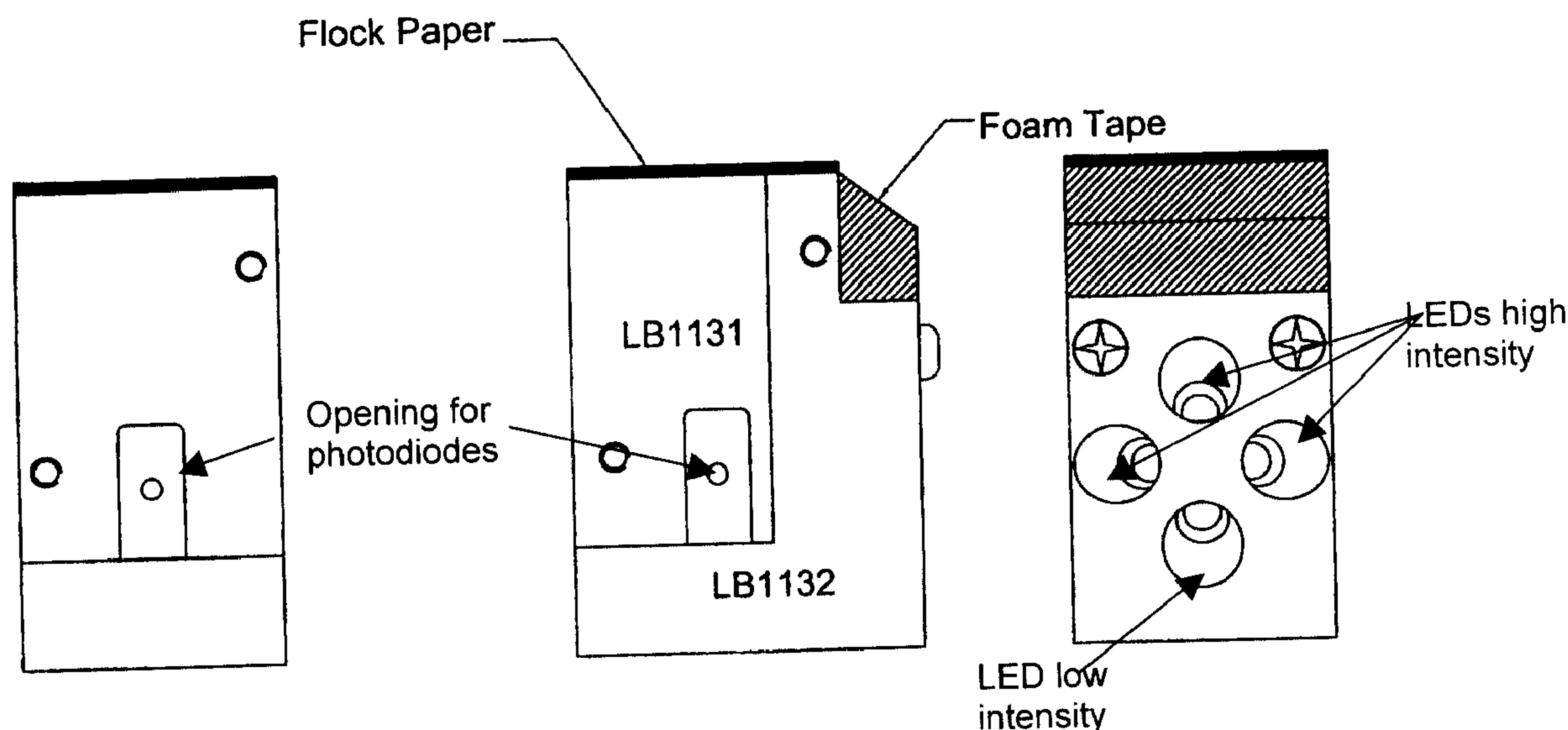
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(54) Titre : METHODE POUR TESTER DES ACTIVITES PHOTOSYNTHETIQUES

(54) Title: METHOD OF TESTING PHOTOSYNTHETIC ACTIVITIES



(57) Abrégé/Abstract:

A method for detecting or quantifying the presence of a toxic molecule in a sample, comprising obtaining stabilised thylakoids membranes, wherein the membranes are stabilised with a ratio chlorophyll: liposomes of at least 10:1 and with PVP, and assessing the photosynthetic efficiency of the thylakoid membranes in the presence of said sample, whereby said molecule is detected when said photosynthetic efficiency is measurably lower in the presence versus in the absence of said sample. The present invention also comprises kits using the stabilised thylakoid membranes for detecting and/or quantifying the molecule in a fluid.



ABSTRACT OF THE DISCLOSURE

A method for detecting or quantifying the presence of a toxic molecule in a sample, comprising obtaining stabilised thylakoids membranes, wherein the membranes are stabilised with a ratio chlorophyll: liposomes of at least 10:1 and with PVP, and assessing the photosynthetic efficiency of the thylakoid membranes in the presence of said sample, whereby said molecule is detected when said photosynthetic efficiency is measurably lower in the presence versus in the absence of said sample. The present invention also comprises kits using the stabilised thylakoid membranes for detecting and/or quantifying the molecule in a fluid.

TITLE OF THE INVENTION

BIOSENSORS, AND METHOD AND KITS FOR USING
SAME

FIELD OF THE INVENTION

[0001] The present invention relates to biosensors, and method and kits for using same. More specifically, the present invention is concerned with photosynthesis-based biosensors, and method and kits for using same.

BACKGROUND OF THE INVENTION

[0002] Many assays for detecting toxic molecules in effluents are inefficient when these molecules are present at very low concentration. Assays that are efficient for this purpose such as those made with living organisms and cells like algae, daphneas, trouts, hepatocytes, or worms are time consuming (up to 72 hours). These methods investigate the presence of toxic substances in water by looking at the survival of these organisms in contact of the water under investigation.

[0003] Assays using the response of photosynthetic organs to toxic molecules have been recently devised which in addition to reducing the response time of the assay to 10 minutes, are advantageously sensitive to molecules affecting photosynthetic electron transport and simple to use. Thylakoids are the organs responsible for photosynthesis in phytoplankton, algae and higher plants. In these assays, the physiological response of thylakoids to stress such as toxic molecules is assessed through indirect measurement of photosynthesis, based on a

functional relationship between photosynthetic efficiency and a stimulated fluorescence signal. The yield of fluorescence by these organs is due to their ability to use light for photosynthesis. Certain methods determine photosynthetic activities from isolated Photosystem II (PS II). For instance, EP patent no. 1,134,585 published on September 19, 2001 in the name of Giardi et al. describes the use of PS II isolated from thylakoids membranes to monitor herbicides. Other methods determine photosynthetic activities from whole plants. For instance, US patent no. 6,121,053 issued on September 19, 2000 in the name of Kolber et al. describes a pulsed fluorometer permitting the measurement of photosynthesis efficiency in plants, phytoplankton and algae. In Laberge (1999) and (2000) are described thylakoids chemically immobilised in a BSA-glutaraldehyde matrix or encapsulated in poly(vinylalcohol) bearing styrylpyridinium substituent groups, respectively. None of these systems have been demonstrated to be stable at room temperature.

[0004] Other methods have used thylakoid membranes themselves which also degrade disadvantageously rapidly at room temperature. Thylakoids samples must be kept at 4°C and even then only last for a few hours (Table 1). The use of thylakoid membranes alone in assays and in particular of *in situ* assays is therefore made awkward. One method sought to remedy this problem by combining whole thylakoid organs (chloroplasts) to membranes.

[0005] An object of the present invention is therefore to provide improved photosynthesis-based biosensors and bioassays for detecting toxic molecules in fluids, and methods and kits for using same.

SUMMARY OF THE INVENTION

[0006] The biosensors methods and kits of the present invention permit to determine the level/presence of certain toxic molecules in fluids including effluents. These biosensors are sensitive to molecules that affect the photosynthetic electron transport. Molecules that can compete with active sites of photosynthetic enzymes and those that have oxydo-reductive properties can therefore be detected with the methods and bioassays of the present invention.

[0007] More specifically, in accordance with the present invention, there is provided biosensors comprising stabilised thylakoid membranes lasting at least 12 days at room temperature.

[0008] There is also provided a method for detecting toxic molecules comprising the use of these biosensors in combination with a fluorometer advantageously permitting the use of microprocessor since the measurement is made with a non-modulated light rather than pulsed light. . The pulsed light permits the measurement of photochemical and non photochemical parameters in the whole plants or algae. In isolated thylakoid membranes, because certain non photochemical parameters can not be obtained, pulsed light may not be necessary. Furthermore, pulsed light fluorometers are relatively complex, costly and bulky apparatuses. Fluorometers using non-modulated light do not have these drawbacks and are nevertheless able to adequately measure the photosynthetic efficiency of thylakoid membranes.

[0009] In accordance with the present invention, there is therefore

provided, a method for detecting or quantifying the presence of a toxic molecule in a sample, comprising obtaining stabilised thylakoids membranes, wherein the membranes are stabilised with a ratio chlorophyll: liposomes of at least 10:1 and PVP; and assessing the photosynthetic efficiency of the thylakoid membranes in the presence of said sample, whereby said molecule is detected when said photosynthetic efficiency is measurably lower in the presence versus in the absence of said sample. In a specific embodiment, the photosynthetic efficiency is assessed with a fluorometer using non-modulated light.

[0010] In accordance with the present invention, there is also provided, a method of stabilising thylakoid membranes for use in bioassays comprising mixing thylakoid membranes with liposomes and with PVP, and wherein a ratio of chlorophyll to liposomes of at least 10:1 is obtained. In accordance with another aspect of the present invention, there is provided a stabilised thylakoid membrane obtained thereby.

[0011] In accordance with the present invention, there is therefore also provided a kit for detecting a toxic molecule comprising thylakoids stabilised with liposomes and PVP. In a specific embodiment, the kit further comprises a portable fluorometer using non-modulated light.

[0012] The terminology “toxic”, “toxic substance” and “toxicity” refers herein to the property of a substance enabling it to inhibit in part or in whole the photosynthetic efficiency of thylakoid membranes.

Fluorescence

[0013] A number of parameters are used to measure the fluorescence emitted by thylakoid membranes. F_0 corresponding to the minimal fluorescence of the activated photosystems. This fluorescence is produced by a weak illumination of the photosystems. F_1 corresponds to an illumination slightly higher than F_0 . F_m corresponds to the fluorescence produced by strong/actinic illumination. Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

[0014] The present invention seeks to meet these needs and other needs.

[0015] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] In the appended drawings:

[0017] Figure 1 schematically illustrates the disposition of LEDs in a fluorometer according to a specific embodiment of the present invention;

[0018] Figure 2 schematically illustrates the measurement mode of a fluorometer according to a specific embodiment of the present invention;

[0019] Figure 3 graphically illustrates the variation of the parameters $F_1(A)$ and $F_m(B)$ as a function of chlorophyll concentration in stabilised thylakoid membranes;

[0020] Figure 4 graphically illustrates the variation of inhibition of the photosynthetic efficiency of stabilised thylakoid membranes by 1,08 $\mu\text{g/mL}$ atrazine according to specific embodiments of the present invention as a function of chlorophyll concentration calculated with the $F_1(A)$ and $F_m(B)$ data presented in Figure 3;

[0021] Figure 5 graphically illustrates the variation of photosynthetic efficiency of stabilised thylakoid membranes according to specific embodiments of the present invention as a function of time after the release of the membranes in a solution;

[0022] Figure 6 graphically illustrates the variation of inhibition of the photosynthetic efficiency of stabilised thylakoid membranes as a function of various concentrations of atrazine, and at different times after their release in solution;

[0023] Figure 7 graphically illustrates the variation of the photosynthetic efficiency of thylakoid membranes as a function of pH;

[0024] Figure 8 graphically illustrates the effect of atrazine on fluorescence parameters F_1 and F_m) and the calculated photosynthetic efficiency with optimal conditions presented in Figures 1-7 on stabilised thylakoid membranes; and

[0025] Figure 9 graphically illustrates the percentage of inhibition by atrazine of stabilised thylakoid membranes determined with a specific embodiment of a fluorometer of the present invention and calculated with the data presented in Figure 8.

DESCRIPTION OF THE PREFERRED EMBODIMENT

[0026] In a specific embodiment, a fluorometer continuously illuminates weakly the photosystems with a light emitting diode (LED) emitting light at a wavelength of 475 or at 660 nm. Better results were obtained with the 475 nm wavelength in order to obtain a higher sensitivity to measure the photosynthetic response to toxic molecules, the intensity of the low excitation light was adjusted in order to obtain a F_1 fluorescence level a little higher than F_0 .

[0027] Fluorescence measured 2 sec after turning on one LED is F_1 . Three additional LEDs are then turned on so as to induce actinic illumination F_m . Dispersion of LEDs are illustrated in figure 1. Light beams are oriented so as to form together a point of convergence precisely at the level where the photodiode is read. After a specific time, chosen according to the nature of the thylakoid membranes used (eg. Source of thylakoids, etc.), of the background noise and of the level of sensitivity sought, the F_m fluorescence is measured. To determine F_1 and F_m , the fluorometer measures wavelengths higher than 650 nm when the illumination is at 475 nm, and higher than 700 nm when illumination is at 660 nm. Figure 2 illustrates how fluorescence measurements are collected and how the fluorometer may be adjusted according to a

specific embodiment of the present invention. The terminology “gain” is used to refer to the signal integration varying as selected from 0.32 to 80 msec. The terminology “Delay” is used in this figure to refer to the delay of 0, 1 to 1000 msec required by LEDs in order to produce the actinic illumination after their activation. The specific delay is adjusted in light of the various parameters listed with regards to the delay before reading. The terminology “strong intensity” is used to refer to the current applied to LEDs to produce a variation in the light intensity between 2 and 20 mV (F_m reading). The terminology “weak intensity” is used to refer to the current applied to the LED to produce a variation in the light intensity between 0 and 1.6 mV (F₁ reading).

[0028] The photosynthetic efficiency is determined with the following formula (F_m - F₁)/F_m. Preferably, the photosynthetic efficiency of the stabilised thylakoids is between 0.7 in order to obtain valid readings. The maximal value would be 0.83 for thylakoids from higher plants and green algae if F₁ was fixed at F₀. Any molecule affecting the photosystems modify either F₁ or F_m which modification necessarily decreases the photosynthetic efficiency. The inhibition curve of a particular molecule is determined by calculating the activity of the photosystems in the absence of the molecule and in the presence of the molecule and comparing both efficiency as follows as described in Conrad 1993:

Without inhibitor (wi) :

$$\text{efficiency}_{(wi)} = (F_{m(wi)} - F_{1(wi)}) / F_{m(wi)}$$

With inhibitor :

$$\text{Relative photosynthetic efficiency}_{(\text{sample})} = (F_{m(\text{sample})} - F_{1(\text{sample})})/F_{m(\text{wi})}$$

Percentage efficiency :

$$\text{efficiency (\%)} = (\text{efficiency}_{(\text{sample})} \times 100) / \text{efficiency}_{(\text{wi})}$$

Percentage inhibition :

$$\text{Inhibition (\%)} = 100 - \text{efficiency (\%)}$$

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Preparation of thylakoid membranes

[0029] All steps are conducted in the dark or under green light and at cold temperature (samples on ice or procedure in cold room). Thylakoid membranes are isolated from 100 g of deveined spinach leaves. Spinach leaves are then crushed in a mixer with homogenising a buffer comprising TES-NaOH 20mM pH 7.5, sorbitol 330mM and MgCL2 5mM. Other conventional buffers could be used including TES, Hepes and Tris. The homogenate is filtered on a cheese cot and the filtrate is centrifuged 2 min at 2500X g at 4°C on a Eppendorf™5810-R, rotor # A-4-44.

[0030] The pellet is then resuspended in a hypotonic solution consisting in 1 in 20 of the homogenising buffer. This step is used to lyse the chloroplast membranes. The resulting solution is then crushed in a Wheaton™ mixer and centrifuged 3 min. at 3500X. The resulting pellet

contain the purified membranes. They are then resuspended in a buffer consisting of TES-NaOH 20 mM pH 7.5, sorbitol 330mM, MgCL₂ 5 mM and NH₄CL 1mM to as to obtain a final concentration of chlorophyll between 2 and 3 mg/ml. The chlorophyll concentration is assessed according to the method described in Porra (1989) described herein. The solution is then either stored at -80°C for later use or may be stabilised for storing at room temperature.

EXAMPLE 2

Stabilisation of thylakoid membranes

[0031] A preparation of 2 to 3 mg/mL of thylakoid membranes were mixed with a phosphate buffer containing 0.02 M pH 6.5, sucrose 300 mM, NH₄Cl 10 mM, MgCL₂ 20 mM, EDTA 10 mM, polyvinylpyrrolidone (PVP) (125 μ L of solution 20% for 1 mL) and liposomes constituted of phosphatidylcholine and phosphatidylglycerol in a ratio of 10:1 to obtain a solution of 0.125 mg/mL. The PVP increased the stability of the thylakoid preparations as compared to the same preparation without PVP. The amount of PVP may be varied without departing from the scope of the invention. Other conventional buffers can also be used without departing from the scope of the present invention.

[0032] The quantity of liposomes used was determined via the ratio chlorophyll/liposome. This ratio may vary and is preferably at least equal to 10:1 and better results with regards to stability and sensitivity were obtained with a ratio 100:1. Any ratio higher than 100:1 is expected to work. The liposomes are believed to help to the formation of thylakoid membranes vesicle thereby increasing their stability and increasing the consistency of the readings.

[0033] 100 μ L of this solution was poured into a 5 mL of 7 mL tube previously refrigerated. The tubes were then frozen and then lyophilised with a speed-vacTM from Savant (#SS-22). Lyophilization was conducted 4 hours in order to remove completely water from the 100 μ L tube. The tubes are then closed and kept at the desired temperature. Table 1 presents the stability of various thylakoid preparations obtained as a function of the temperature of storage. Table 2 presents the detection sensitivity of thylakoid membranes stabilised according to the method presented herein as well as their median inhibitory concentration (IC 50) on various herbicides. In some of the thylakoid membranes preparations of the present invention, a detection threshold of 1 ppb was observed with atrazine.

Table 1 : Stability of various thylakoid membranes preparation

T°	Conservation time			
	Natives	PVP	PVP (under vacuum)	PVP (under vacuum + liposomes)
- 80 °C	> 5 month	> 5 month	> 5 month	N.D.
- 20 °C	> 3 month	> 3 month	> 3 month	N.D.
4 °C	18 hours	14 days	> 1 month	N.D.
20 °C	4 hours	7 days	11 days	12 days
37 °C	N.D.	2 days	4 days	4 days

Table 2: Detection sensitivity of stabilised thylakoid membranes and median inhibitory concentration (IC 50) on various herbicides

Analyte	Detection sensitivity (mg/L)	CI 50(r2) confidence interval 95 % (mg/L)	Quebec Water quality Norms	
			Potable water (mg/L)	Chronic Toxicity (mg/L)
Atrazine	0,002	0,728 (0,9987) 0,716-0,739	0,005	0,0018
Diethyl atrazine	0,013	n. d.	0,005	n. d.
Desiopropyl atrazine	0,126	22,6 (0,9592) 19,9-25,3	0,005	n. d.
Cyanazine	< 0,001	0,04 (0,9847)	0,01	0,002

		0,038-0,042		
Diuron	0,002	0,071 (0,8816) 0,068-0,074	0,15	0,016
Metolachlor	n.d.	n.d.	0,05	0,0078
2,4-D	n.d.	n.d.	0,1	0,047

[0034] The measurement of photosynthetic activity of the stabilised thylakoid membranes according to the methods of the present invention is validated with the dichlorophenol (DCIP) reduction method.

EXAMPLE 3

Determination of thylakoid membrane activity by the DCIP reduction method

[0035] Test tube A: To 750 μ L of a blank buffer (Sodium phosphate 0,5 M pH 6,2) or of a lyophilisation buffer (phosphate of sodium 0,5, M pH 6,2 containing EDTA 10 mM; $MgCl_2$ 20 mM; NH_4Cl 10mM; sucrose 330 mM; PVP 1%), add 500 μ L of DCIP solution 3,6 mM, and 500 μ L of the thylakoid dispersion (Thylakoid membranes diluted to 17,5 μ g/ mL⁻¹ of chlorophyll with ice-cold blank buffer or lyophilization buffer).

[0036] Test tube B: to 750 μ L of the blank buffer (buffer (Sodium phosphate 0,5 M pH 6,2) or of the lyophilization buffer (phosphate of sodium 0,5, M pH 6,2 containing EDTA 10 mM; $MgCl_2$ 20 mM; NH_4Cl 10mM; sucrose 330 mM; PVP 1%), add 500 μ L of DCIP solution 3,6 mM and 500 μ L of distilled water.

[0037] Then read the absorbance at 655 nm for both tubes. Incubate 10 minutes under a 100 W lamp at room temperature and read again the absorbance at 655 nm. The activity is calculated with the following formula : (Reading at 655 nm after illumination) – (reading at

655 nm after illumination).

[0038] The results are expressed as DPIP reduction per mg of chlorophyll per hour. Different volume of DPIP solution are diluted in appropriate volume of buffer and the absorbance is read at 655 nm.

Table 3 Concentration/volume of DPIP used relating to volume of buffer used

Concentration of DPIP mM	Volume of DPIP 3,6 mM (μ L)	Vol. of blank or lyophilised buffer (mL)
0	0	1,75
0,05	103	1,65
0,10	206	1,54
0,30	309	1,44
0,40	412	1,34
0,60	618	1,13
0,70	721	1,03
0,90	927	0,82

Table 4 Absorbance of solution before and after illumination

Concentration of DPIP mM	Absorbance at 655 nm (before illumination)	Absorbance at 655 nm (after illumination)
0	0	0
0,05	0,125	0,125
0,10	0,218	0,216
0,30	0,545	0,535
0,40	0,875	0,871
0,60	1,259	1,249
0,70	1,311	1,302
0,90	1,878	1,874
Sample 0,45; 0,46	Sample 2,009; 1,932	Sample 1,145; 0,981

EXAMPLE 4

Measure of chlorophyll concentration in stabilised thylakoid membranes

[0039] The chlorophyll concentration in the stabilised preparation is measured with a conventional absorbance dosage test. 10 μ L of

thylakoids extract is added into 5 mL Aceton 80%. The mixture is then centrifuged 5 min on a table centrifuge, and the absorbance is read at 647 nm and at 664 nm on a photometer. The total chlorophyll concentration is then calculated with the following formula as described in Porra (1989): $(17,76 \times A_{647}) + (7,34 \times A_{664}) \times 500 / 1000 = \text{mg/mL}$.

[0040] The chlorophyll concentration is then adjusted by diluting the preparation with a suitable buffer or water to reach the desired concentration. In a specific embodiment, this concentration is of 5 $\mu\text{g/mL}$.

EXAMPLE 5

Use of stabilised thylakoid membranes of the present invention

[0041] Before use, the tubes containing stabilised thylakoid membranes are retrieved from storage. If they were stored in the freezer or the refrigerator, they are tempered 30 minutes at room temperature and in the dark. Two millilitre and a half of water is added to 100 μL of the thylakoids preparation at a concentration 5 $\mu\text{g/mL}$. The tubes are then shook by inversion for 2 min. The tubes are then left in the dark for a further 10 min before fluorescence measures are taken.

EXAMPLE 6

Comparison of stability of photosynthesis-based biosensors of the prior art with stabilised thylakoid membranes according to the present invention

Table 5 Comparison of detection sensitivity of photosynthesis-based biosensors of the prior art with that of the present invention

Detected substance	Threshold detection (mg/L) (detection mode)				
	Stabilised thylakoid membranes of the present invention (fluorescence)	Chemically immobilised Thylakoids ^a (electrochemistry)	PSII screen printed electrode ^b (electrochemistry)	PSII Clark electrode ^b (amperometry)	algae (fluorescence) ^c
Atrazine	0,002	0,01-0,05	0,0004	0,0004	0,009
Diuron	0,002	0,01	0,0002	0,0001	0,002
Immobilised preparation stability T _{1/2} at 22°C	> 265 hours	50 hours ^f	24 hours ^b	24 hours ^b	No immobilisation

T_{1/2} = The time required for the photosynthetic activity of the thylakoid membranes to decrease to half of its initial value.

Table 6 Comparison of median inhibitory concentrations (IC₅₀) of photosynthesis-based biosensors of the prior art on various herbicides with those of the present invention

Substance tested	IC ₅₀ (mg/L)						
	Thylakoids			PSII		algae	
	Stabilised thylakoid membranes of the present invention (fluorescence)	Chemically immobilised thylakoids ^a (electrochemistry)	Physically immobilised ^d (electrochemistry)	Screen printed electrode ^b (electrochemistry)	Clark electrode ^b (amperometry)	native ^c (fluorescence)	native ^e (fluorescence)
Atrazine	0.728	0.33	0.28	0.019	0.065	0.062	0.15
Cyanazine	0.04	0.079	0.31				
Diuron	0.071	0.12	0.13	0.16	0.019	0.012	0.959
2,4 D	n. d.	> 1000					
Metolachlor	n. d.	580					
Immobilised preparation stability T _{1/2} at 4°C	> 720 hours*	800 hours ^f	2160 hours ^g	Not available	Not available	No immobilisation	No immobilisation

T_{1/2} = The time required for the photosynthetic activity of the thylakoid membranes to decrease to half of its initial value.

^a Laberge (1999);

^b Koblizek, (2002);

^c Jay (1997);

^d Laberge (2000) ;

^e Conrad (1993);

^f Loranger (1994);

^g Rouillon (2000) ;

Table 3 The loss of efficiency of native membranes after lyophilisation as compared to that obtained after physical immobilisation is

of 20% and 40%, respectively.

EXAMPLE 7

Determination of IC₅₀ of stabilised thylakoid membranes with atrazine

Table 7 Linear regression Log-Probit values between 5 and 95 % effect

atrazine mg/L	Log(10) Conc. *10	% effect	probit
4	1.602	64.8	5.377
0.4	0.602	45.7	4.892
0.04	-0.398	24.6	4.313
0.004	% -1.398	10.6	3.752
0.001	% -2.000	5.3	3.383

Slope (B) = 0.56; Data number = 5 ; Interception = 4.52; degree of freedom = 3 ;Variance (S²) = 0.001; standard error = 0.034; Standard error of regression line = 0.015; correlation coefficient @ = 0.9994 ; correlation coefficient for a confidence of 95% = 0.878; determination coefficient (R²) = 0.9987; regression formula : Y = 4.52 + 0.556 * X; regression formula: X = Y – 4.521/ 0.556; CI₅₀ = 0.728 mg/L (0.716-0.739); Slope significant value: T = 48.1 dl = 3 p = 0.0002;

This line is significantly different from 0. Its confidence interval can be calculated as: 0.556 +/- T(3) * 0.011

EXAMPLE 8

Effect of time on fluorescence emission by stabilised thylakoid membranes

[0042] The incubation time before taking fluorescence measurement was fixed at ten minutes. Fluorescence emission up to 60 minutes after thylakoids release in solution is substantially similar to that found at ten minutes. It is believed that Measuring after less than 10 minutes after release may affect the sensitivity of the tests since the lower

concentration of toxic molecules takes more time to inhibit the fluorescence signal.

EXAMPLE 9

Effect of concentrations of chlorophyll in stabilised thylakoid membranes preparation on fluorescence measurement

[0043] The variation of the parameters $F_1(A)$ and $F_m(B)$ was determined as a function of chlorophyll concentration (Figure 3) on membranes stabilised with PVP and liposomes as described herein. These measurements determined the lowest effective concentration of membrane thylakoids (expressed as chlorophyll concentration) needed to perform the test. Concentrations between 5 and 10 $\mu\text{g/mL}$ of chlorophyll thylakoids give almost the same result. Using the lowest chlorophyll concentration possible is desirable to limit the turbidity of the solution caused by chlorophyll which decreases fluorescence. Figure 4 illustrates that a lower chlorophyll concentrations, better results are obtained.

EXAMPLE 10

Effect of pH on fluorescence measurement on stabilised thylakoid membranes

[0044] The variation of the photosynthetic activity of thylakoid membranes as a function of pH was measured on thylakoids stabilised with PVP and liposomes as described herein. The results presented in Figure 7 indicates that basic pHs (defined herein as higher than 7,8) inhibit photosynthetic efficiency as do toxic substances do. Therefore, in specific embodiments of the method of the present invention, the pH of the water solution, is adjusted at 6,7 prior to releasing the thylakoid

membranes for fluorescence measurement.

EXAMPLE 11

Sensitivity of F_1 and F_m measures as compared to photosynthesis efficiency to measure inhibition

[0045] The ability of the measures F_1 and F_m to provide accurate information on the presence of a toxic agent was compared to that provided by the formula $(F_m - F_1)/F_m(wi)$ (photosynthesis efficiency). The measures were performed on thylakoid membranes stabilised with PVP and liposomes as described herein. As is illustrated in Figure 9, photosynthesis efficiency formula $(F_m - F_1)/F_m(wi)$ is more sensitive than F_1 and F_m to evaluate the effect of inhibitors.

[0046] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

REFERENCES

1. Conrad, R.; C. Büchel; C. Wilhelm; W. Arslane; C. Berkaloff and J.-C. Duval "Changes in yield of in-vivo fluorescence of chlorophyll as a tool for selective herbicide monitoring" (1993) *J. Appl. Phycol.* 5; 505-516;
2. Porra R.J., Thompson W.A., Kriedemann P.E., (1989) *Biochimica Biophysica Acta* 975, 384-394;
3. Ministère de l'Environnement du Québec (2001). *Critères de la qualité de l'eau de surface au Québec*. Direction des écosystèmes aquatiques, Ministère de l'Environnement, Québec;
4. Laberge, Chartrand, Rouillon, Carpentier (1999). "In vitro phytotoxicity screening test using immobilized spinach thylakoids" *Env. Tox. Chem.* 18 (12): 2851-2858 ;
5. Koblizek, Maly, Masojidek, Komenda, Kucera, Giardi, Mattoo, Pilloton (2002). "A Biosensor for the Detection of Triazine and Polylurea Herbicides Designed Using Photosystem II Coupled to a Screen-Printed Electrode" *Biotechnol. Bioeng.* 78 (1): 110-116;
6. Jay, Ducruet, Duval, Pelletier (1997). "A high-sensitive chlorophyll fluorescence assay for monitoring herbicide inhibition of photosystem II in the chlorophyte *Selenastrum capricornutum*": Comparison with effect on cell growth. *Arch. Hydrobiol.* 140 (2): 273-286;
7. Laberge, Rouillon, Carpentier (2000). "Comparative study of thylakoid membranes sensitivity for herbicide detection after physical or chemical immobilization" *Enz. Microb. Technol.* 26: 332-336 ;
8. Loranger, Carpentier (1994) "A fast bioassay for phytotoxicity measurements using immobilized photosynthetic membranes" *Biotech. Bioeng.* 44:1-5.
9. Rouillon, Carpentier (2000) "Amperometric activity measurements of photosynthetic material immobilized in poly(vinylalcohol)-SbQ Application to detect pollutants" *Curr. Topics Electroch.* 7: 125-133.

WHAT IS CLAIMED IS:

1. A method for detecting or quantifying the presence of a toxic molecule in a sample, comprising
obtaining stabilised thylakoids membranes, wherein the membranes are stabilised with a ratio chlorophyll: liposomes of at least 10:1 and PVP; and
assessing the photosynthetic efficiency of the thylakoid membranes in the presence of said sample,
whereby said molecule is detected when said photosynthetic efficiency is measurably lower in the presence versus in the absence of said sample.
2. A method as recited in claim 1, wherein the photosynthetic efficiency is assessed with a fluorometer using non-modulated light.
3. A method of stabilising thylakoid membranes for use in bioassays comprising mixing thylakoid membranes with liposomes and with PVP, and wherein a ratio of chlorophyll to liposomes of at least 10:1 is obtained.
4. A stabilised thylakoid membrane obtained according to the method recited in claim 3.
5. A kit for detecting a toxic molecule comprising thylakoids stabilised with liposomes and PVP.

6. A kit as recited in claim 5 further comprising a portable fluorometer using non-modulated light.

Application number / numéro de demande: 2412206

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10^{ème} étage)

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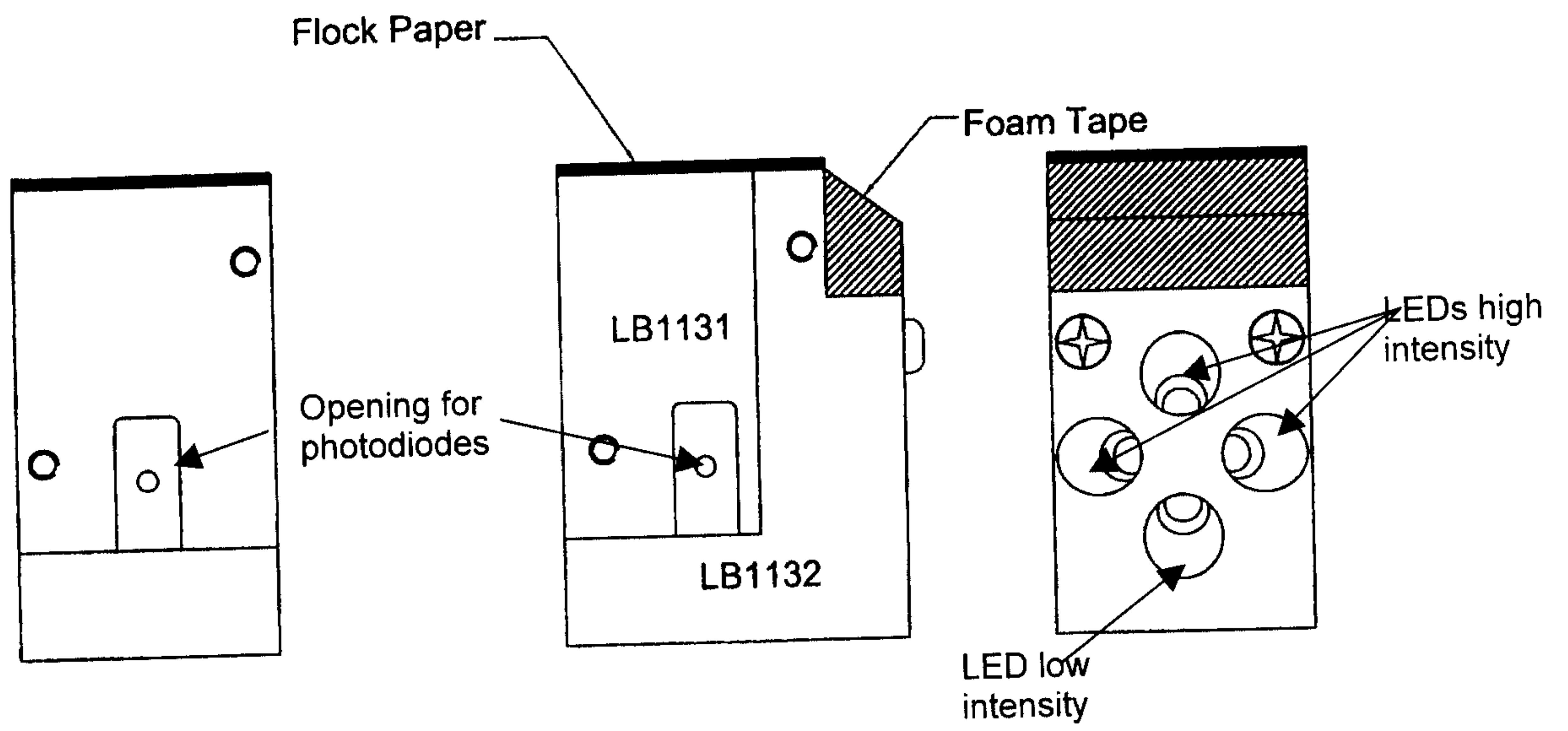


Figure 1

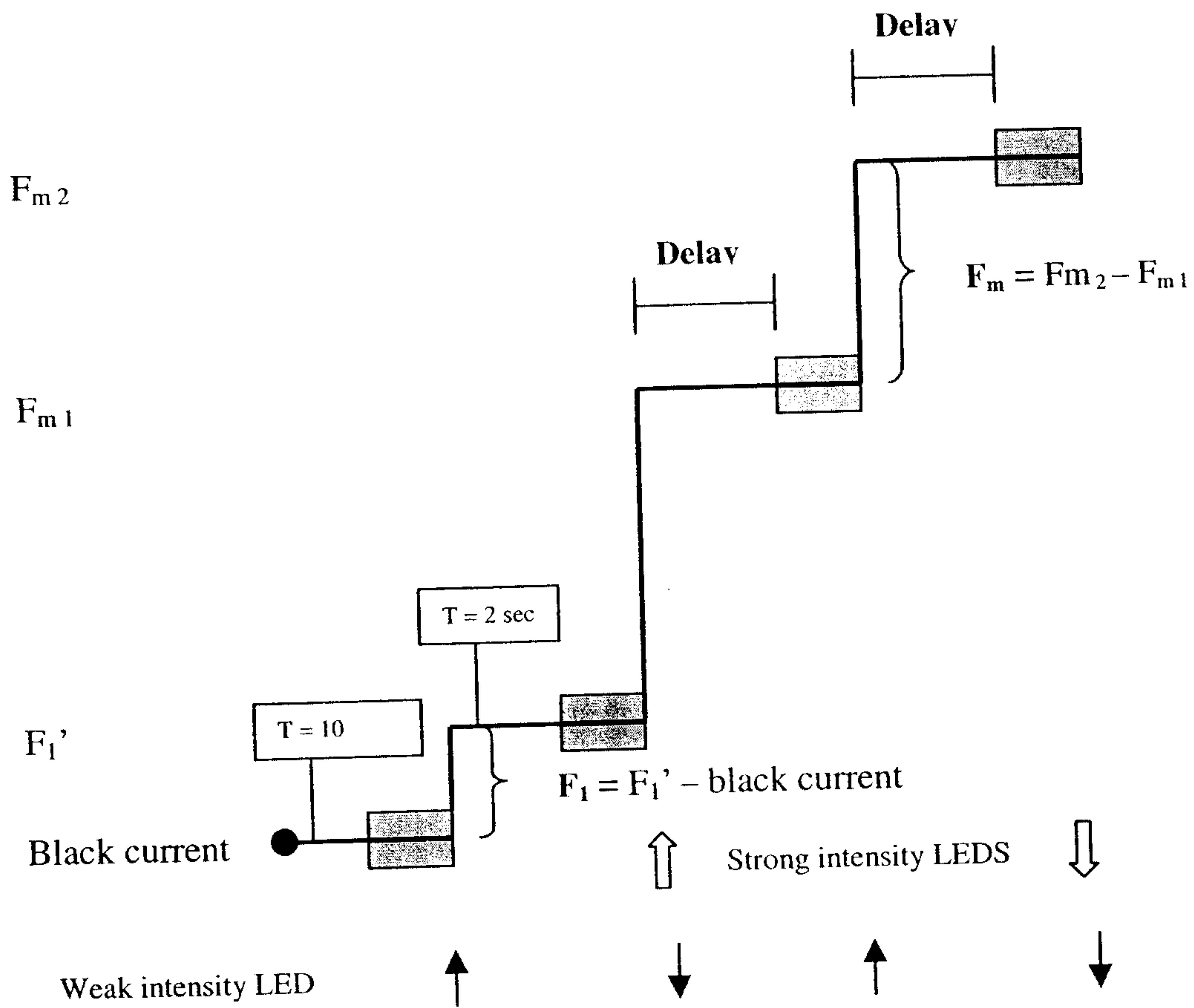


Figure 2

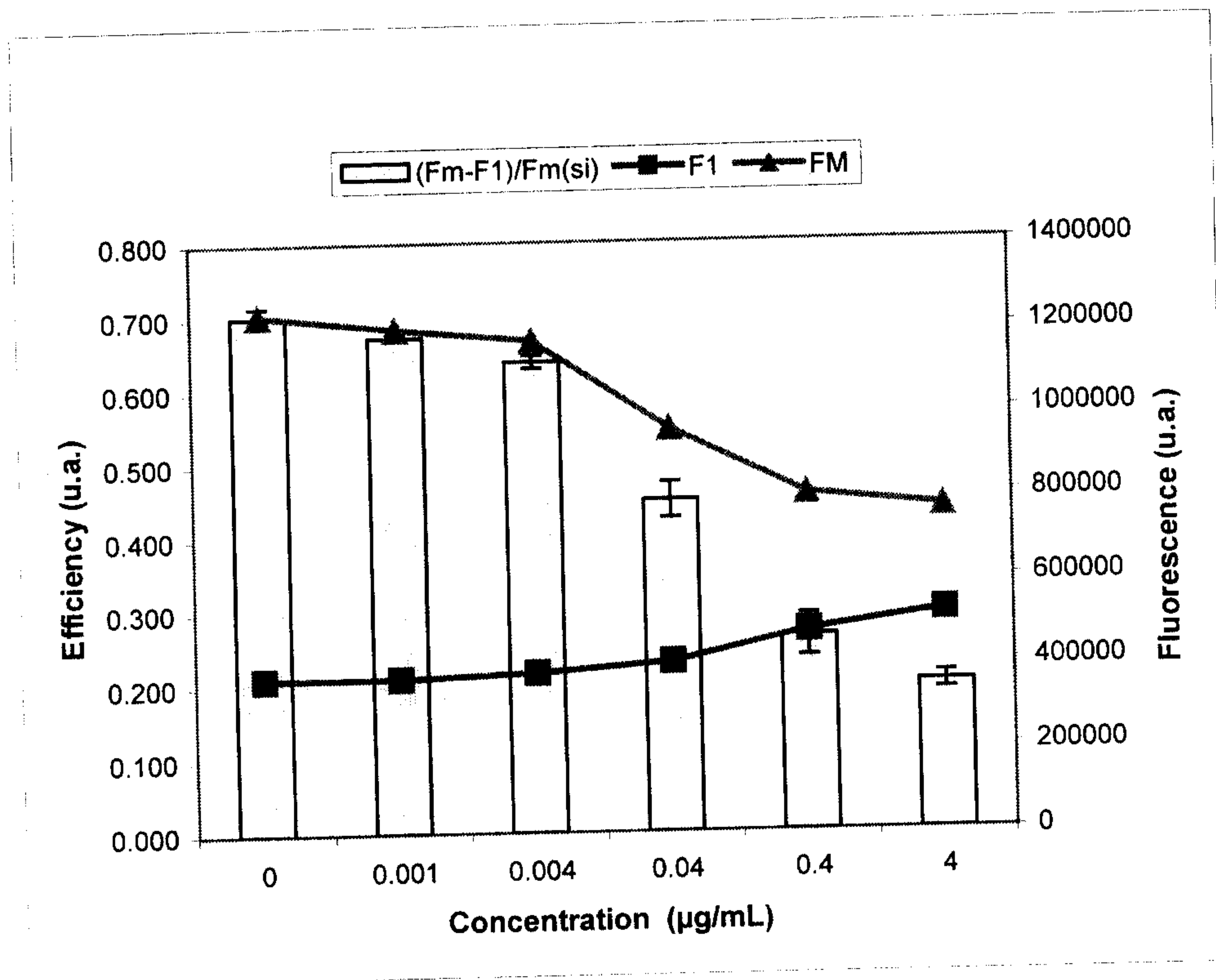


Figure 8

30

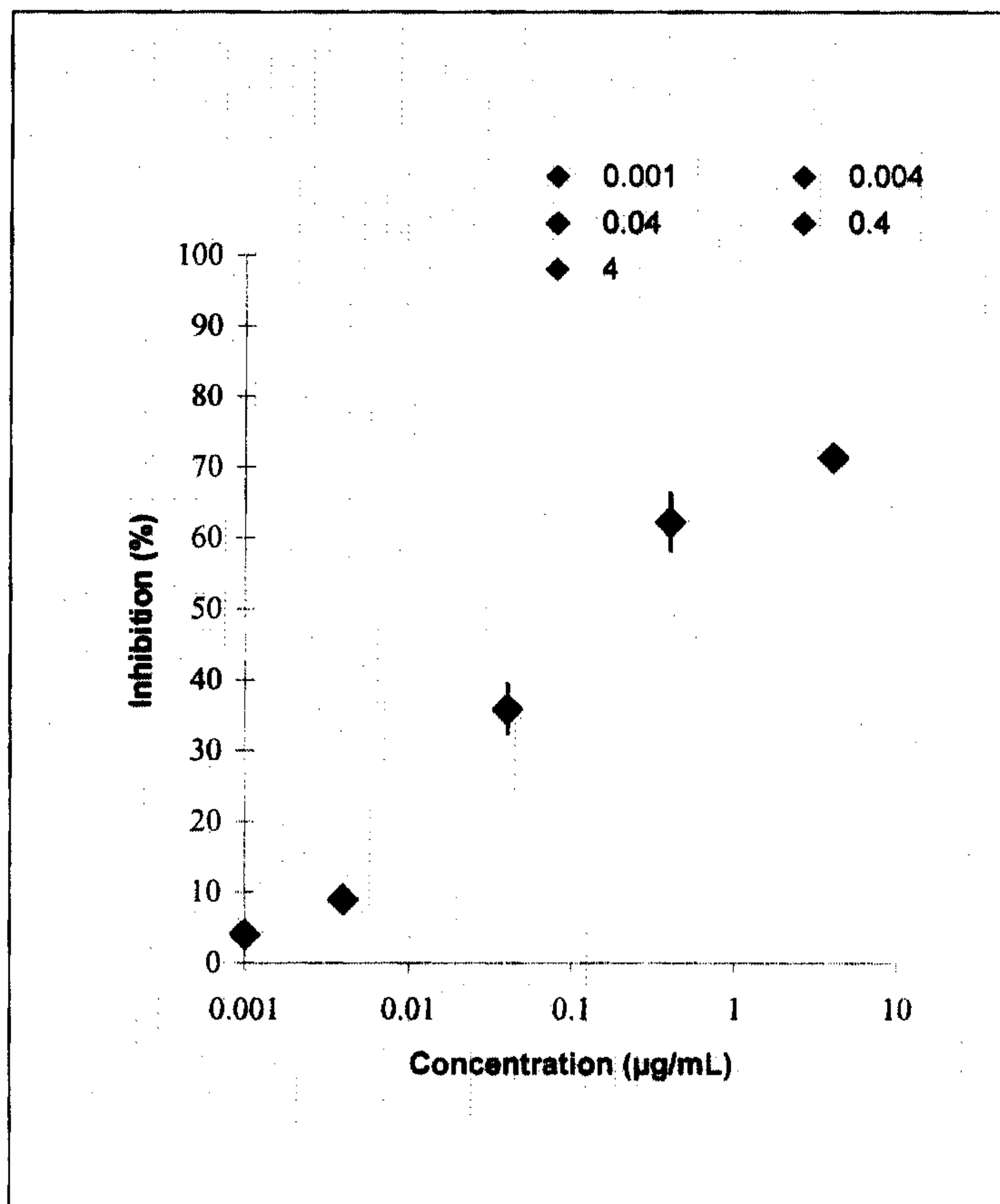
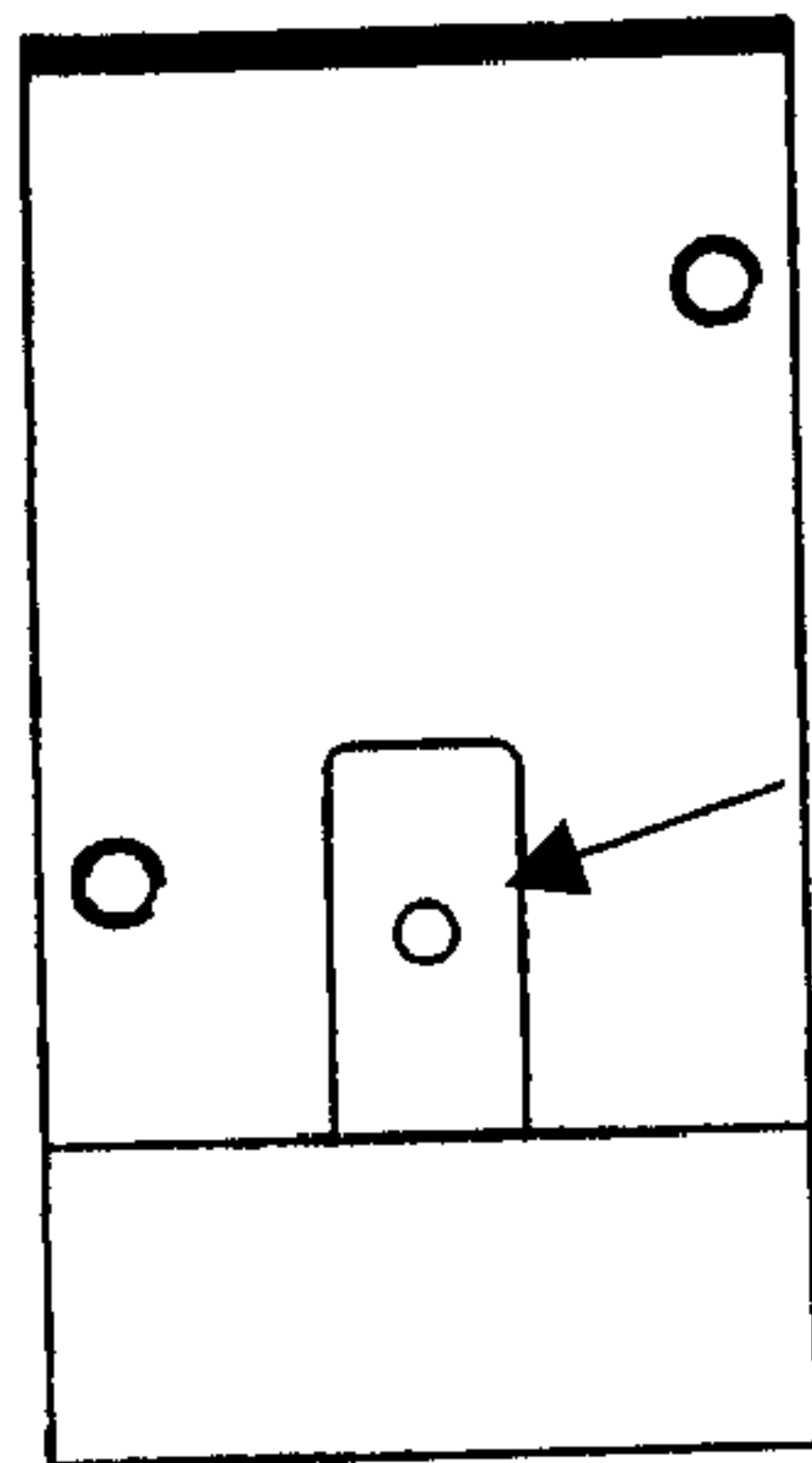


Figure 9

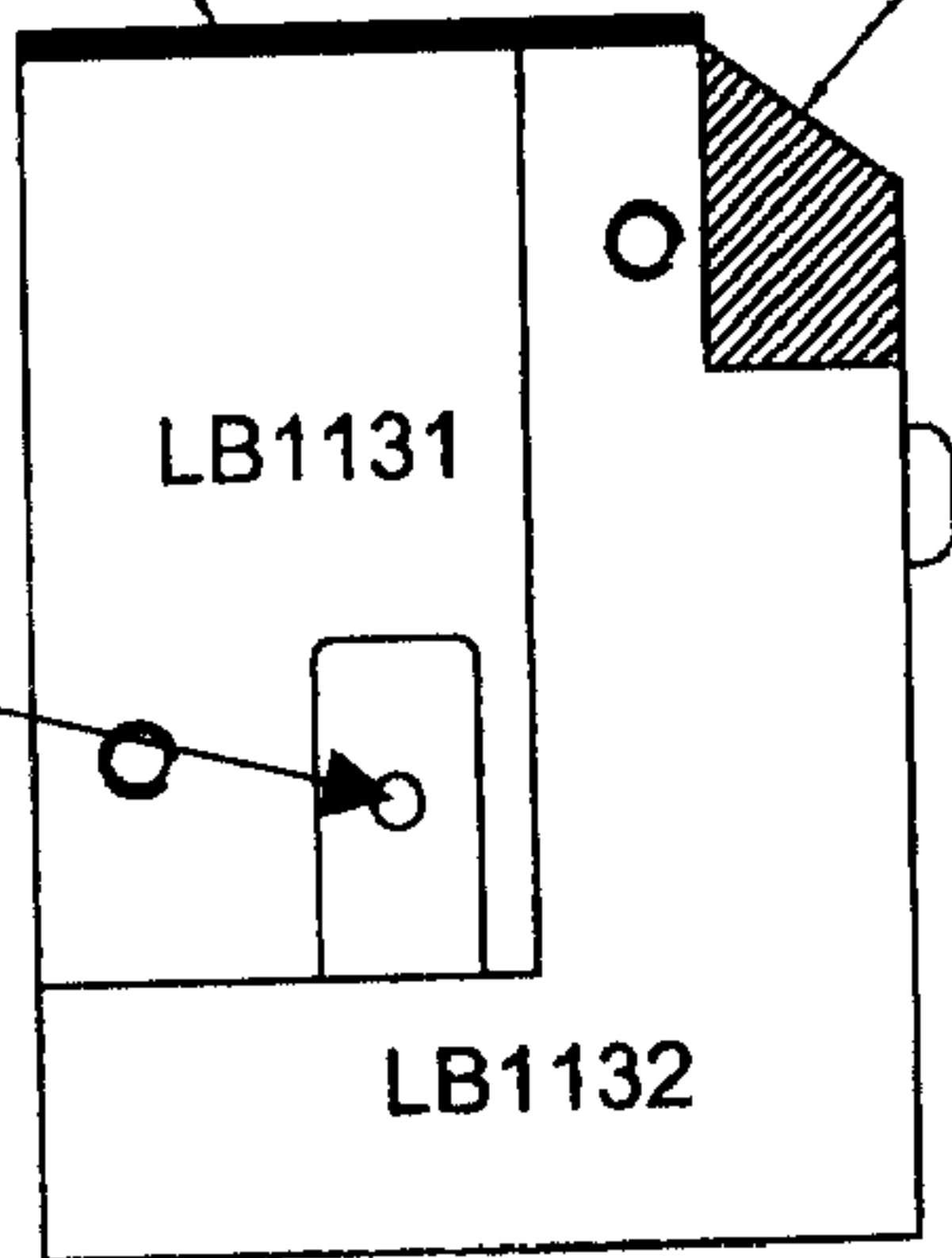
Flock Paper



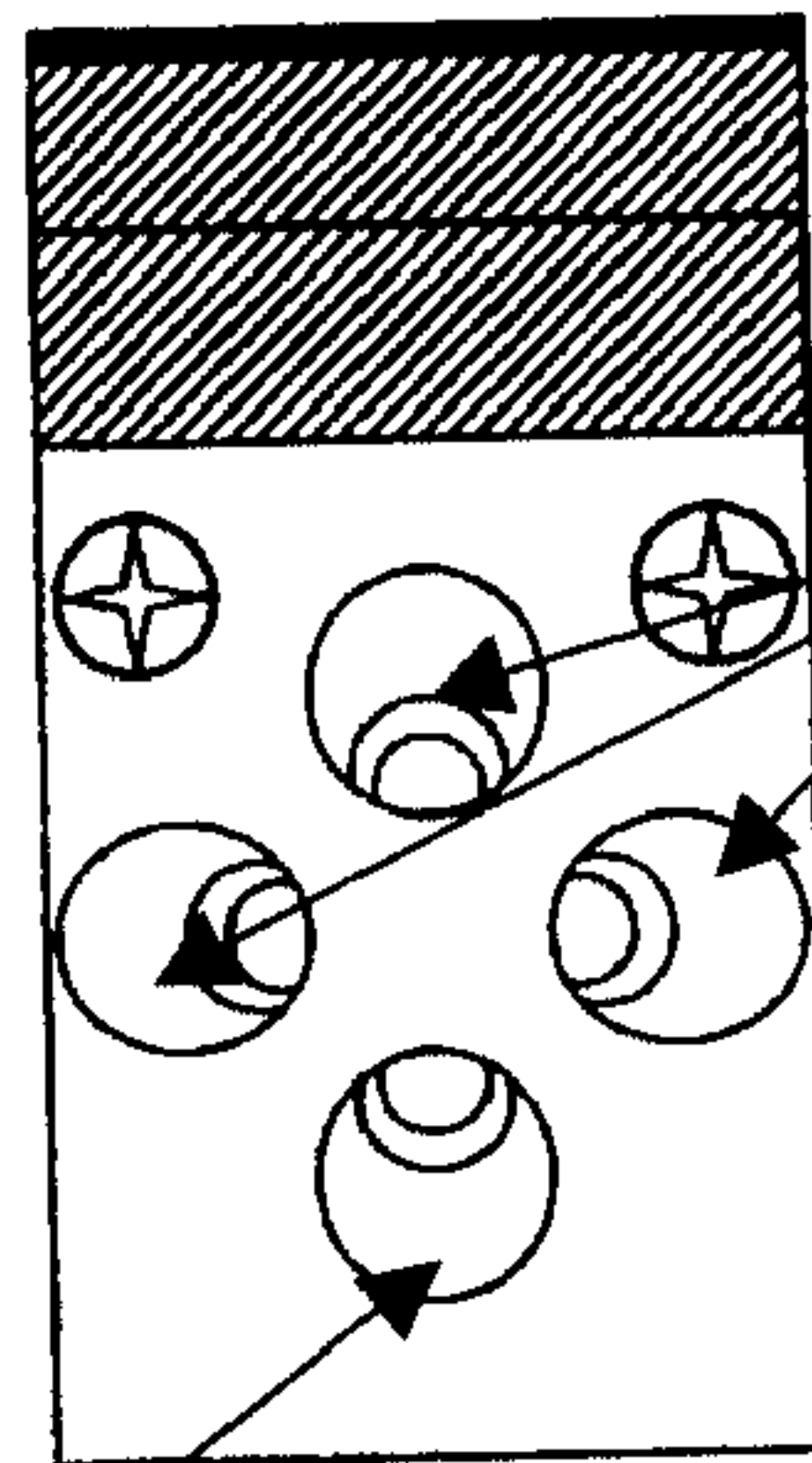
Opening for
photodiodes

LB1131

LB1132



Foam Tape



LEDs high
intensity

LED low
intensity