流式细胞仪在水生生物研究中的应用

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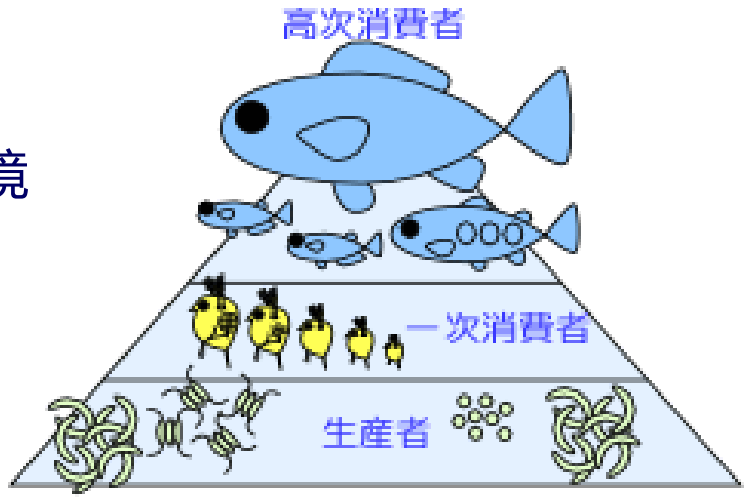
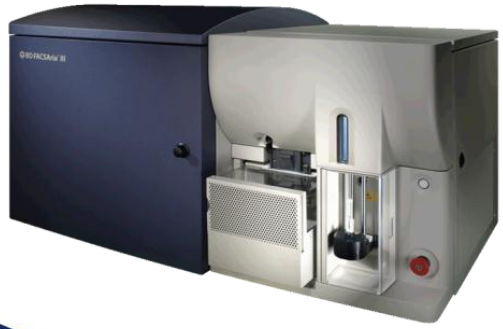
水生生物

- 水生生物：种类繁多
- 研究意义：水生生物学、水生生态学、水体环境
- 传统的研究方法：



- **流式细胞仪与水生生物的研究**：
  1. 快速准确地对样本中的单细胞或某一细胞类群进行分析；
  2. 完成对复杂组分中特定细胞类群的获取，为后续的研究奠定基础。

主要内容



- 水体微型生物
- 水生动物
- 环境毒理

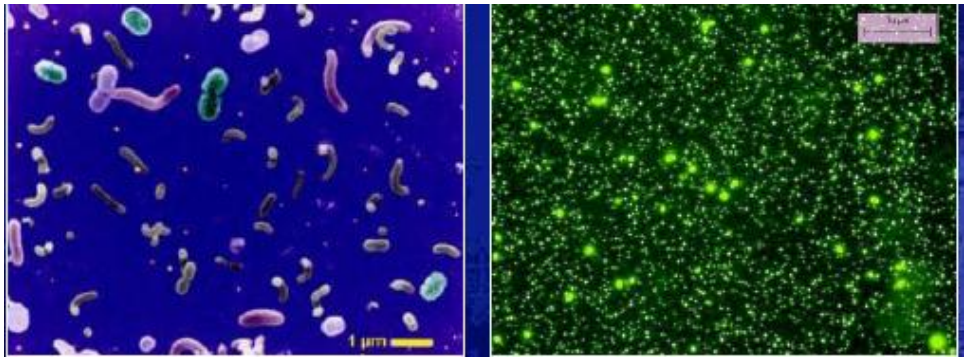
水体中的微型生物——“看不见的巨人”

数量巨大：

- 超微型真核自养生物：100 cells/mL - 1,000 cells/mL
- 聚球藻：1,000 cells/mL - 10,000 cells/mL
- 原绿球藻：10,000 cells/mL -100,000 cells/mL
- 浮游细菌：100,000 cells/mL - 1,000,000 cells/mL
- 浮游病毒：1,000,000 cells/mL - 10,000,000 cells/mL

个体微小：

- ❖ **超微型浮游生物：0.2-2μm**
- ❖ **纳米级浮游生物：2-20μm**
- ❖ **微米级浮游生物：20-200μm**



Whitman *et al*., *PNAS*, 1998; Karl, *Nature*, 2002

水中微型生物的特点

- 个体微小：纳米-微米级别；
- 数量巨大：每一滴海水中有100万个细菌；
- 种类繁多：仅培养的不到1%；
- 功能复杂：参与所有物质和能量循环；
- 分布广泛：各种极端环境均有发现。

研究手段？

1. 个体较小，常规显微观察困难；
2. 工作量大，操作繁琐；
3. 显微计数主观，结果不准；
4. 性质功能检测受限制。

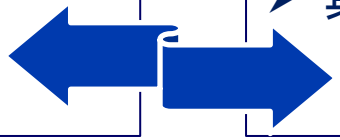
流式细胞仪技术优势

以其样品制备简单、可进行快速多参数数据采集、测定精确、不易受溶解有机物的干扰等优越性而成为水生和环境微生物学研究的重要工具。

根据微粒的荧光特性反映出浮游微型生物的大小、形状、结构或者是色素类型，从而对浮游微型生物进行定量和定性研究。对单个细胞进行分选，方便对不同种浮游生物进行分离和富集培养。

水体中微型生物特点：

- 个体微小
- 数量巨大
- 水中悬浮性
- 种类繁多
- 功能复杂
- 分布广泛



流式细胞仪特点：

- 灵敏度高；
- 分析速度快；
- 精确度高；
- 多参数分析；
- 具有分选功能；

流式细胞术在水体研究中的应用策略

样本采集

- 河流湖泊

样本处理

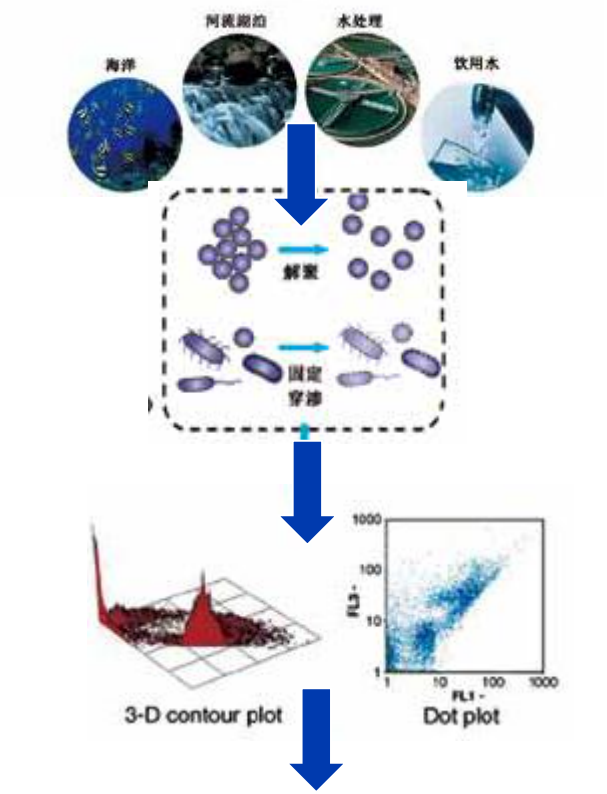
- 细胞群解聚（振荡/超声）
- 预过滤（15-35um滤器）
- 细胞固定/染色标记

流式分析

- 固有属性
- 荧光染色

流式分选

- 获取目标群体



流式在水体微型生物研究中的应用

- 病毒和细菌的检测
  - 计数
  - 活力检测
  - 分类鉴定
- 藻类
  - 水体藻类的分类和多样性研究
  - 水体藻类的活性和功能检测
  - 水体藻类分选应用

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  - 水体藻类分选应用

1. 计数

- **水源中细菌的研究：**基本的需要是菌体计数；
- **常规计数方法：**

①平板法培养法；②通用的显微技术

局限性：误差大、耗时长，且平板法只能计数活菌

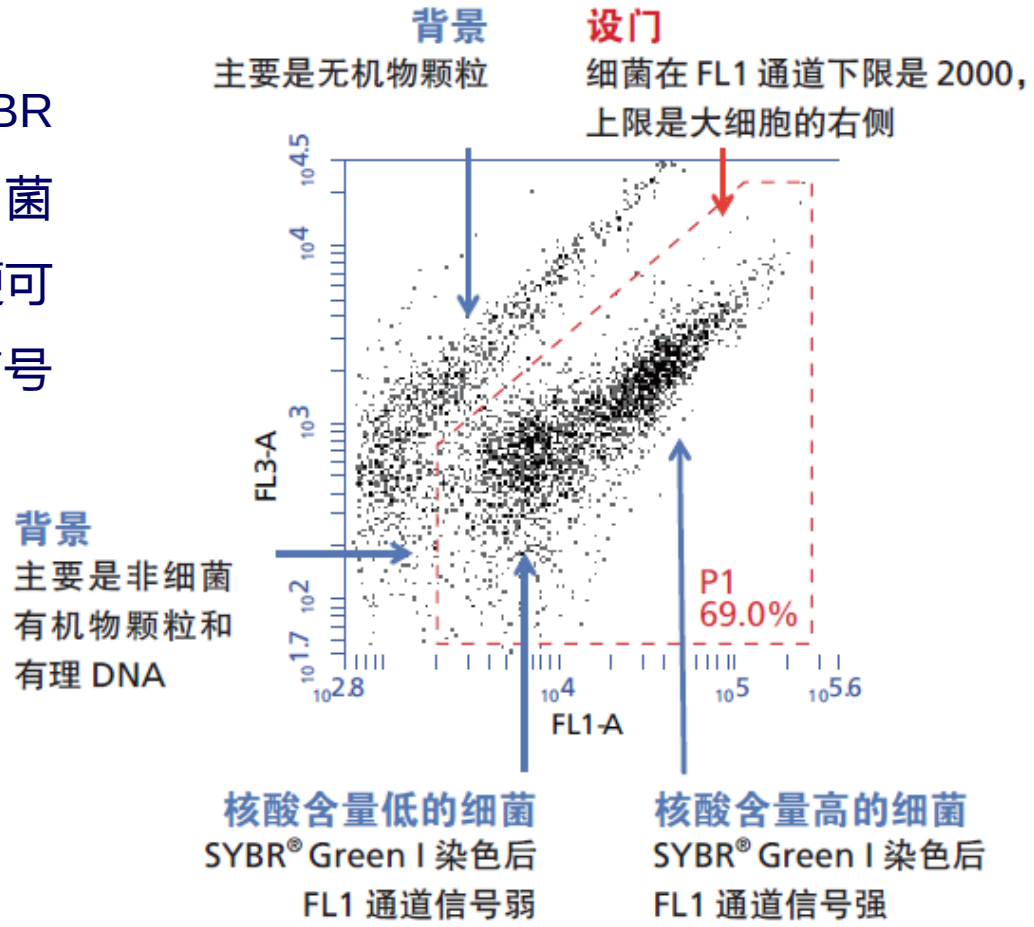
- **流式细胞仪用于菌体计数：**

1) 快速得到细菌总数；
2) 若检测的是一定体积样品中的菌数，即得知菌浓度；
3) 可区分活菌和死菌，获得活菌百分比。

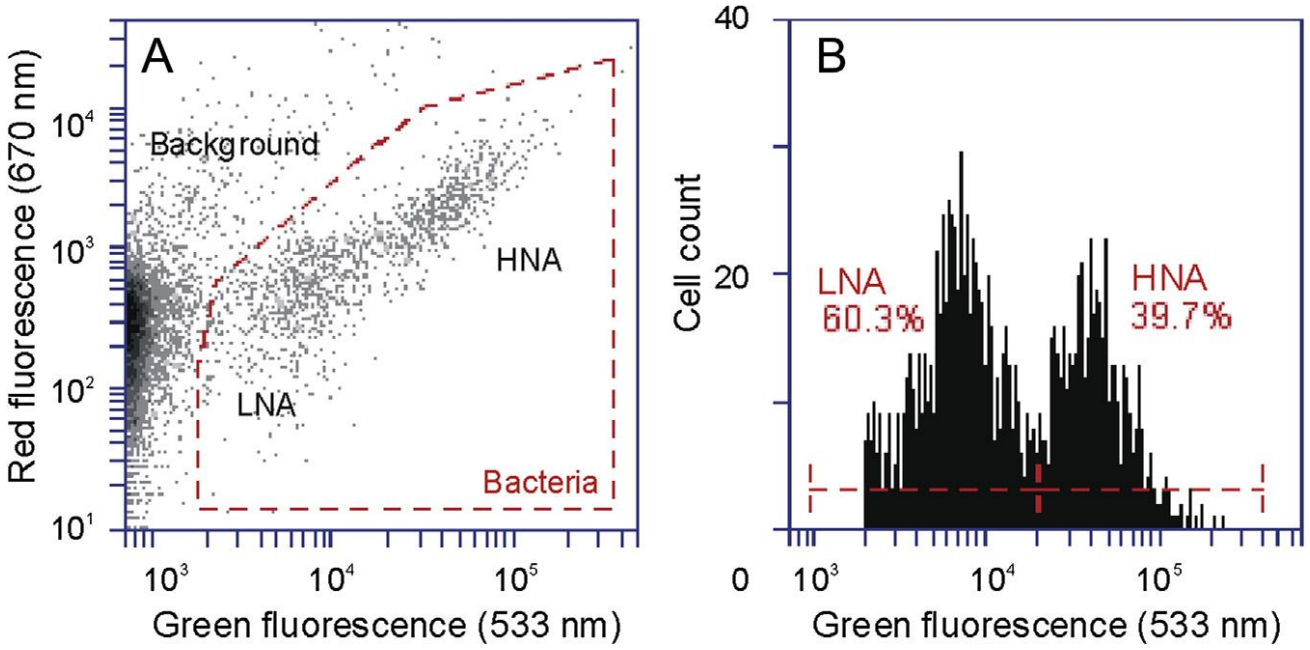
1. 计数

流式检测原理：

利用合适的核酸荧光染料（如SYBR Green I，SYTOX Green I 等）对细菌 DNA 进行染色后，使用流式细胞仪便可以对这些微小的微生物细胞从背景信号中检测出来，并进行分群。



应用举例



绿色荧光/细胞数一维直方图，显示LNA（低核酸含量）和HNA（高核酸含量）细菌聚类及比例统计。

Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method

E.I. Prest, F. Hammes, et al. Water Research (2013) Vol.47: 7131-7142.

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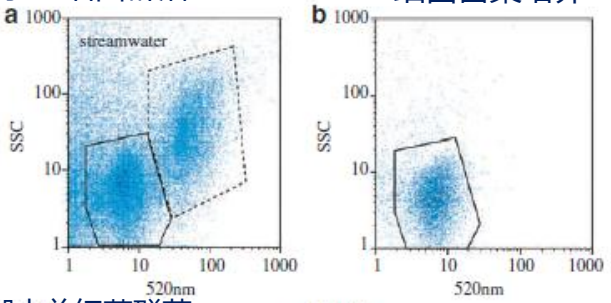
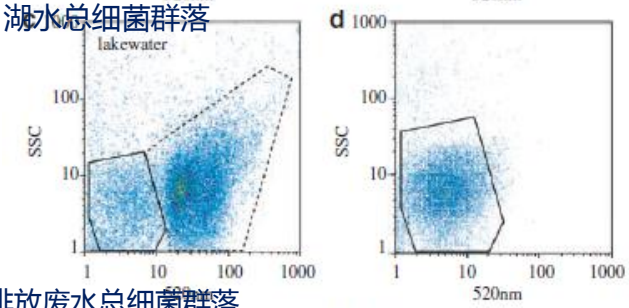
应用举例

溪水总细菌群落　　LNA细菌富集培养



湖水总细菌群落



排放废水总细菌群落

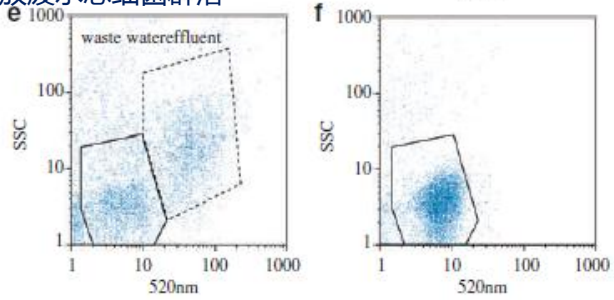


Table 1 Percentage of LNA bacteria in total bacterial communities from different aquatic environments

Source	*DOC* ($mg\,l^{-1}$)	*Total cell concentration* (10^6 $cells\,ml^{-1}$)	*Percentage of LNA*
Chriesbach stream	3.0 ± 0.3	3.21 ± 0.20	68% ± 2%
Alpine stream	0.5 ± 0.1	0.10 ± 0.00	74% ± 5%
Tap water	0.7 ± 0.1	0.15 ± 0.01	53% ± 1%
Groundwater	0.5 ± 0.2	0.31 ± 0.02	75% ± 3%
Lake Greifensee	3.2 ± 0.3	2.41 ± 0.23	23% ± 5%
Wastewater effluent	7.0 ± 0.5	10.6 ± 1.20	58% ± 2%

Results are shown in the format of average ± standard deviation calculated from at least three samples for each source location.

图：水样经SYBR Green I染色处理，流式细胞仪分析结果显示总细菌群落主要有低核酸含量（LNA）和高核酸含量（HNA）细菌两群；而LNA细菌富集培养后占主导。

表：**显示的是不同水环境中LNA细菌占总细菌群落的百分比。**

Isolation and characterization of low nucleic acid (LNA)-content bacteria

Yingying Wang, Frederik Hammes, et al. ISME Journal (2009) Vol. 3: 889-902.

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 02 June 2014
doi: 10.3389/fmicb.2014.00265

The feasibility of automated online flow cytometry for *in-situ* monitoring of microbial dynamics in aquatic ecosystems

*Michael D. Besmer[1,2], David G. Weissbrodt[1,3], Bradley E. Kratochvil[1], Jürg A. Sigrist[1], Mathias S. Weyland[1] and Frederik Hammes[1]**

[1] Department of Environmental Microbiology, Eawag – Swiss Federal Institute for Aquatic Science and Technology, Dübendorf, Switzerland
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瑞士联邦水产科学技术研究所已经开发出一种标准的流式细胞仪染色方法，用以区分饮用水样品中的细菌和碎片。

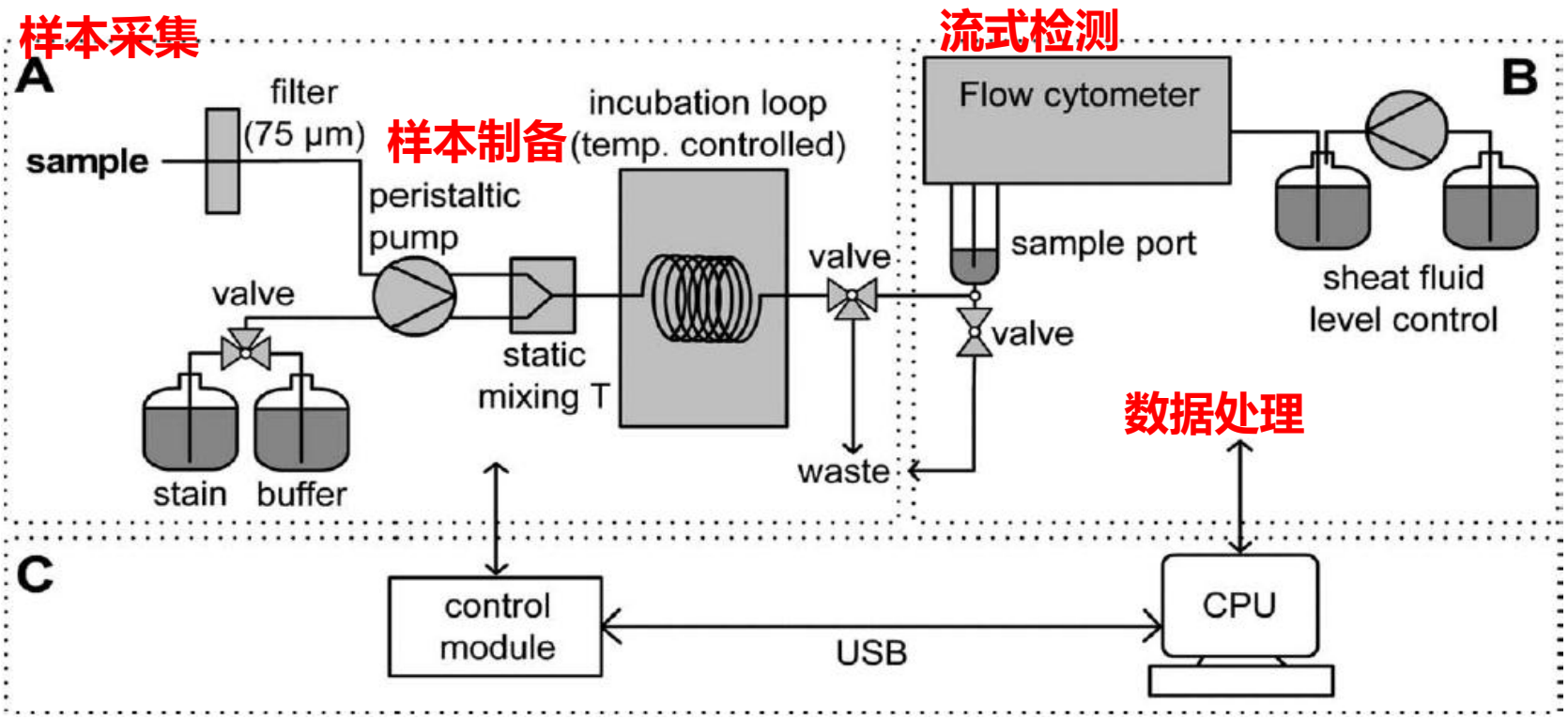


Table 1 | Overview of measured parameters and employed measurement devices for each water type.

Measured parameters	Tap water	River water	Measurement device	Figure/Table
Total cell concentration	x	x	Online FCM	**Figures 1–6**
Percentage of LNA content bacteria	x	x	Online FCM	**Figures 1–6**
Conductivity		x	Online sensor	**Figures 5, 6**
Temperature		x	Online sensor	**Figures 5, 6**
pH		x	Online sensor	**Figures 5, 6**
Oxygen concentration		x	Online sensor	**Figures 5, 6**
Rainfall volume		x	Weather station [NABEL (FOEN and EMPA)]	**Figures 3, 5**

TCC/Days LNA/Days (14 days)

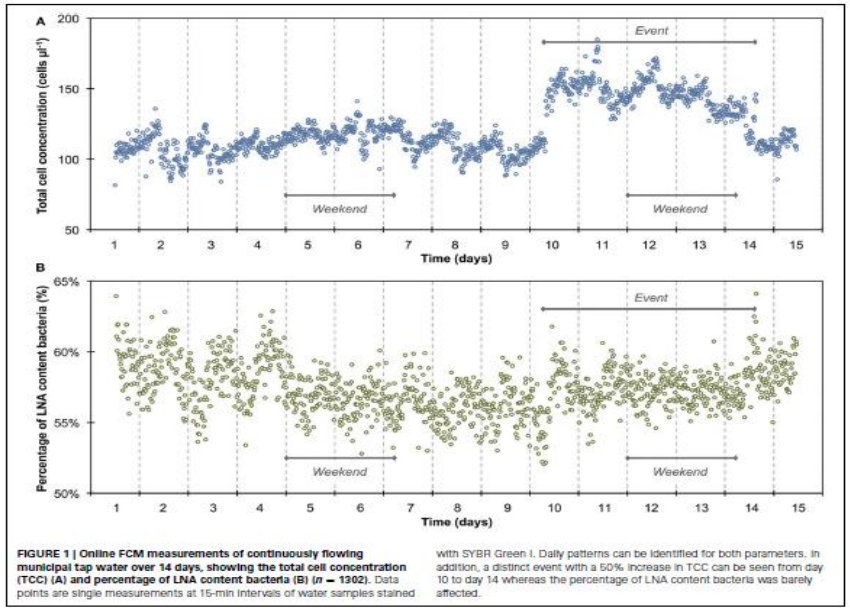


FIGURE 1 | Online FCM measurements of continuously flowing municipal tap water over 14 days, showing the total cell concentration (TCC) (A) and percentage of LNA content bacteria (B) ($n = 1302$). Data points are single measurements at 15-min intervals of water samples stained with SYBR Green I. Daily patterns can be identified for both parameters. In addition, a distinct event with a 50% increase in TCC can be seen from day 10 to day 14 whereas the percentage of LNA content bacteria was barely affected.

TCC/Time LNA/Time (One day)

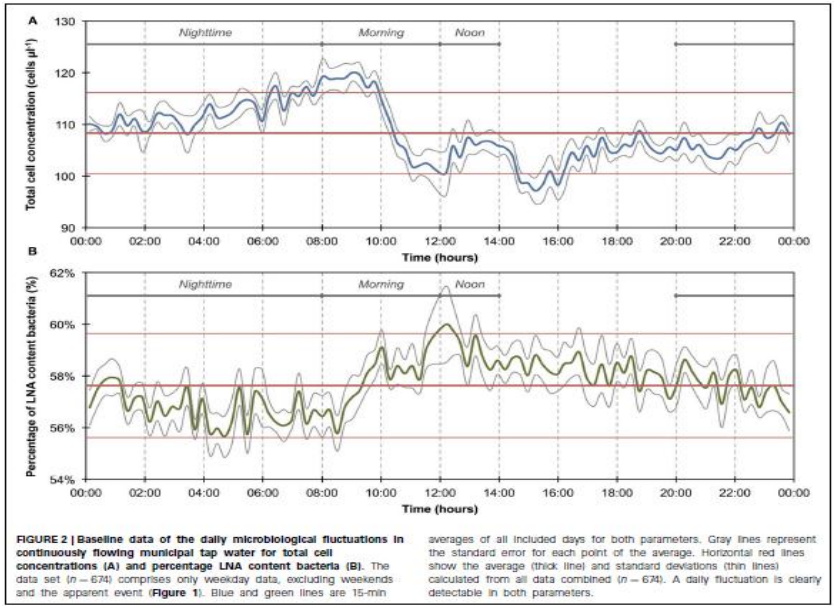
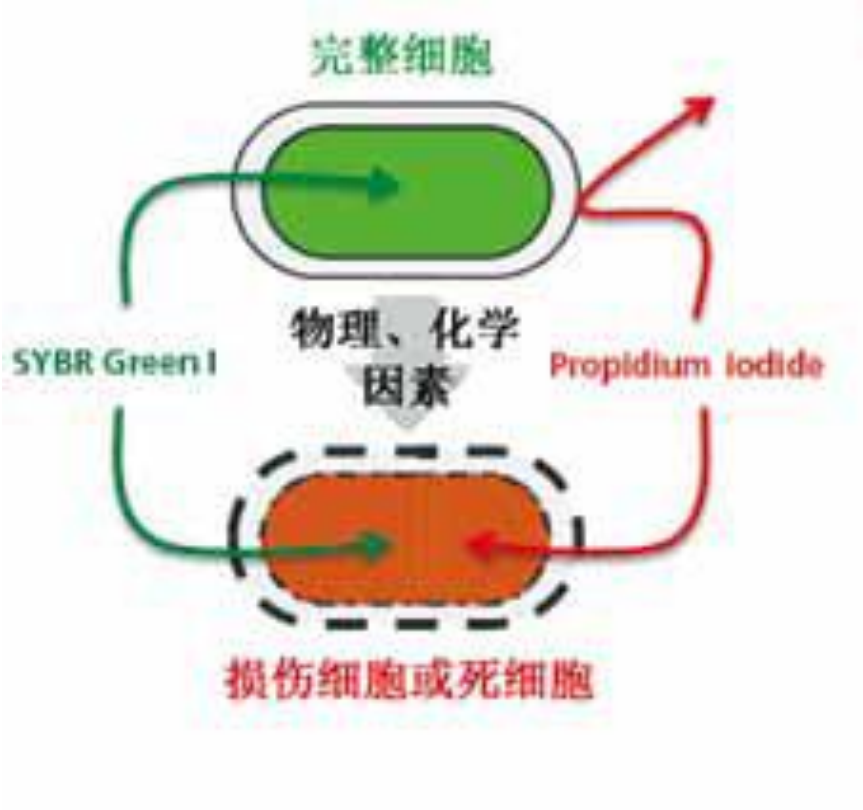
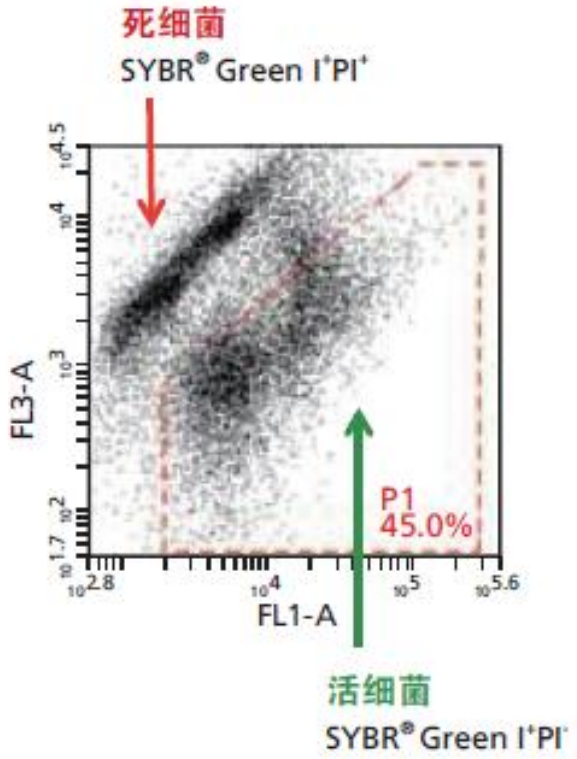


FIGURE 2 | Baseline data of the daily microbiological fluctuations in continuously flowing municipal tap water for total cell concentrations (A) and percentage LNA content bacteria (B). The data set ($n = 674$) comprises only weekday data, excluding weekends and the apparent event (**Figure 1**). Blue and green lines are 15-min averages of all included days for both parameters. Gray lines represent the standard error for each point of the average. Horizontal red lines show the average (thick line) and standard deviations (thin lines) calculated from all data combined ($n = 674$). A daily fluctuation is clearly detectable in both parameters.

2. 活力检测

- 原理：SYBR Green I/PI 双染色检测



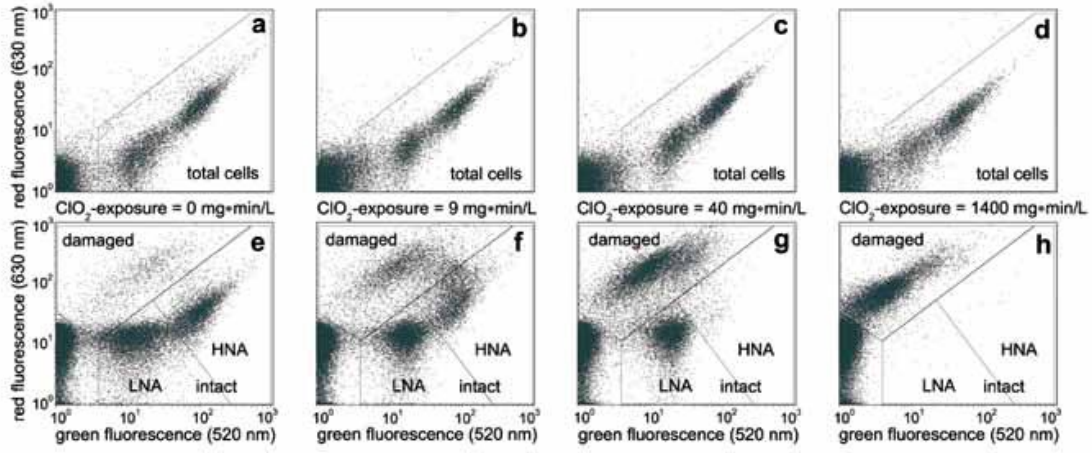


应用举例：快速评估物理化学作用对细菌细胞活性的影响

强氧化剂处理对水中细菌活力影响

摘自：**Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate (VI), and permanganate**

Maaike K. Ramseier, Urs von Gunten, et al. Water Research (2011) Vol. 45: 1490-1500.



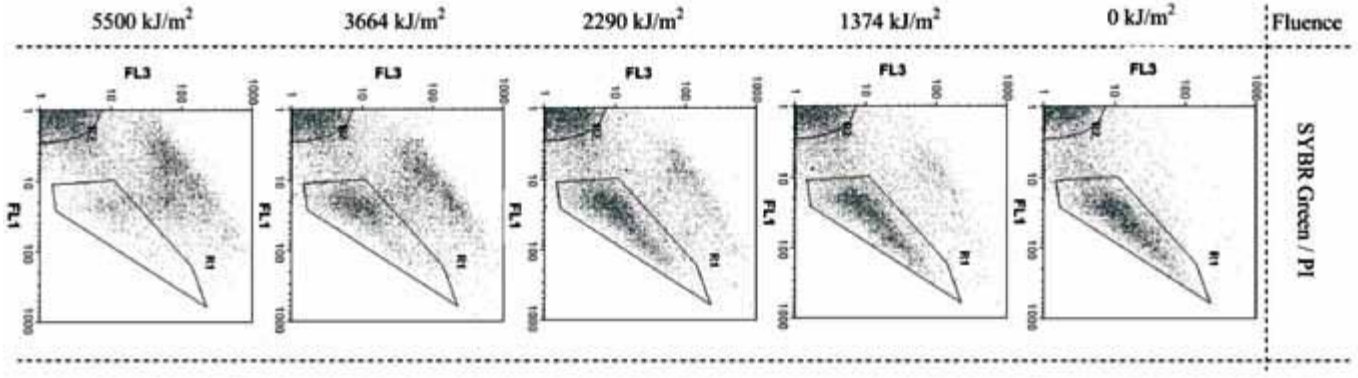
图释：流式细胞术分析不同浓度的二氧化氯对于水样中细菌活力的损伤作用。(a-d). SYBR Green I (SGI)染色；(e-h). SGI与PI双染色。总细胞=完整细胞+损伤细胞(SGI染色阳性)。

结果显示：二氧化氯处理可以增加水样中损伤细胞的比例，且呈现剂量依赖。

UVA 射线对细菌活力影响

摘自：**Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry**

Michael Berney, Frederik Hammes, et al. Applied and Environmental Microbiology (2007) Vol. 73: 3283-3290.



图释：流式细胞术分析人造UVA射线对水样中细菌活力损伤作用。结果表明，人造UVA射线可以降低水样中细菌的细胞活力，并呈浓度依赖关系。

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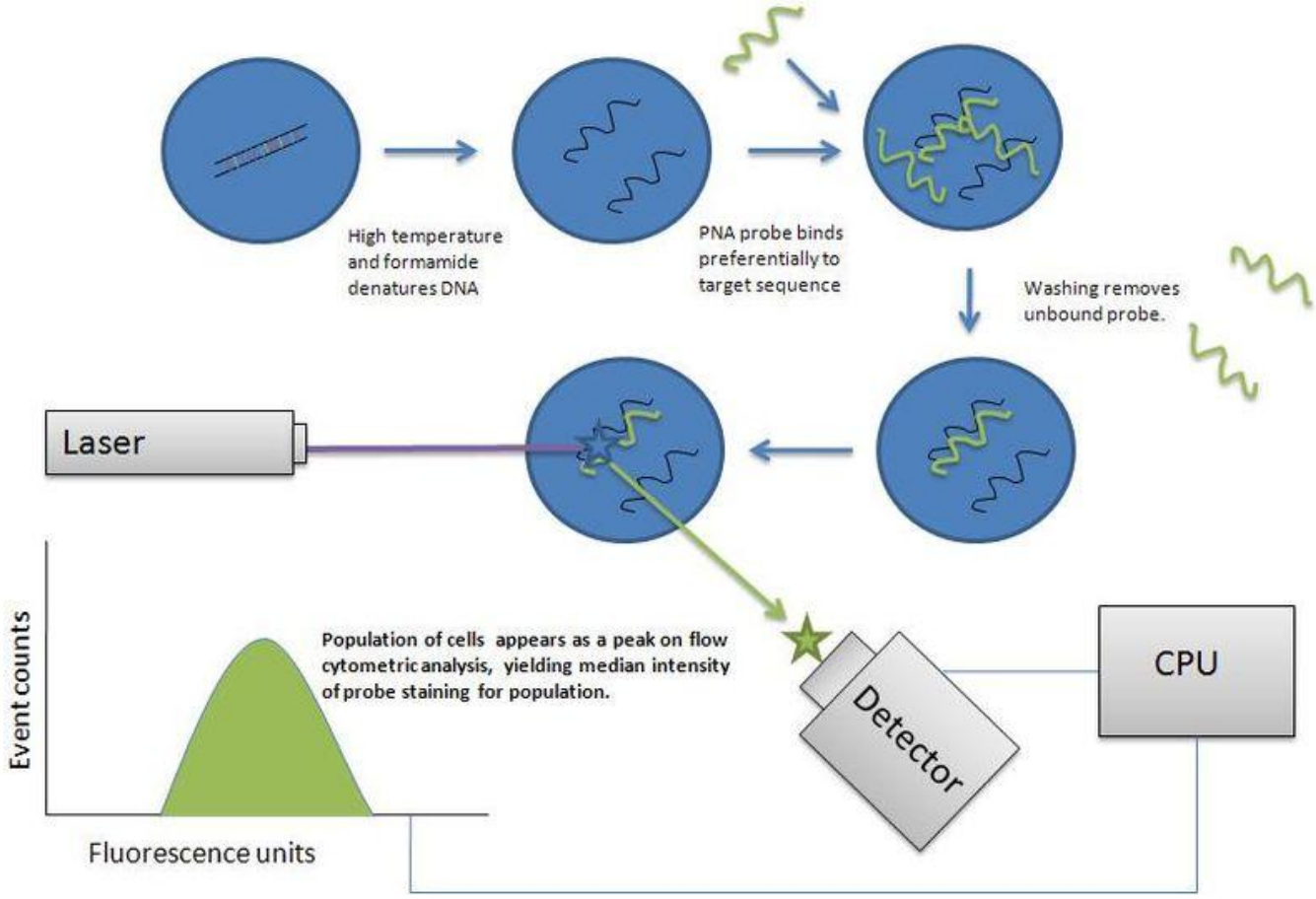
3. 分类鉴定

利用流式细胞术- 荧光原位杂交技术(Flow cytometry - fluorescent in situ hybridization，FCM-FISH)，可以快速对样本中的特定类群的微生物进行分类鉴定，并可以分选进行后期分析。



种群特异的DNA序列，以利用荧光标记的特异寡聚核苷酸片段作为探针，与环境基因组中DNA分子杂交检测该特异微生物种群的存在与丰度。

应用举例

Flow Sorting of Marine Bacterioplankton after Fluorescence In Situ Hybridization

Raju Sekar, Bernhard M. Fuchs, Rudolf Amann, and Jakob Pernthaler*

Max Planck Institute for Marine Microbiology, Bremen, Germany

Received 8 March 2004/Accepted 31 May 2004

We describe an approach to sort cells from coastal North Sea bacterioplankton by flow cytometry after in situ hybridization with rRNA-targeted horseradish peroxidase-labeled oligonucleotide probes and catalyzed fluorescent reporter deposition (CARD-FISH). In a sample from spring 2003 >90% of the cells were detected by CARD-FISH with a bacterial probe (EUB338). Approximately 30% of the microbial assemblage was affiliated with the *Cytophaga-Flavobacterium* lineage of the *Bacteroidetes* (CFB group) (probe CF319a), and almost 10% was targeted by a probe for the β-proteobacteria (probe BET42a). A protocol was optimized to detach cells hybridized with EUB338, BET42a, and CF319a from membrane filters (recovery rate, 70%) and to sort the cells by flow cytometry. The purity of sorted cells was >95%. 16S rRNA gene clone libraries were constructed from hybridized and sorted cells (S-EUB, S-BET, and S-CF libraries) and from unhybridized and unsorted cells (UNHYB library). Sequences related to the CFB group were significantly more frequent in the S-CF library (66%) than in the UNHYB library (13%). No enrichment of β-proteobacterial sequence types was found in the S-BET library, but novel sequences related to *Nitrosospira* were found exclusively in this library. These bacteria, together with members of marine clade OM43, represented >90% of the β-proteobacteria in the water sample, as determined by CARD-FISH with specific probes. This illustrates that a combination of CARD-FISH and flow sorting might be a powerful approach to study the diversity and potentially the activity and the genomes of different bacterial populations in aquatic habitats.

Probe	Sequence (5′ → 3′)	Target organisms	Reference	Hybridized fraction (%)[c]
EUB338	GCT GCC TCC CGT AGG AGT	Domain *Bacteria*	2	91 ± 1.3
NON338	ACT CCT ACG GGA GGC AGC	Complementary to EUB338	2	<1
ALF968	GGT AAG GTT CTG CGC GTT	Most α-proteobacteria	17	26.7 ± 1.4
ROS537	CAA CGC TAA CCC CCT CC	*Roseobacter* spp. and SAR83 α-proteobacteria	12a	11.0 ± 0.1
BET42a[b]	GCC TTC CCA CTT CGT TT	β-Proteobacteria	28a	9.3 ± 3.1
OM43–162	ATG CGG CAT TAG CTA ACC	OM43 clade of β-proteobacteria	This study	4.0 ± 0.2
Nso190	CGA TCC CCT GCT TTT CTC C	Ammonia-oxidizing β-proteobacteria	31	3.4 ± 0.2
Nso1225	CGC CAT TGT ATT ACG TGT GA	Ammonia-oxidizing β-proteobacteria	31	4.0 ± 0.4
GAM42a[b]	GCC TTC CCA CAT CGT TT	γ-Proteobacteria	28a	20.0 ± 1.4
SAR86–1245	TTA GCG TCC GTC TGT AT	SAR86 cluster of γ-proteobacteria	55a	15.2 ± 0.2
CF319a	TGG TCC GTG TCT CAG TAC	*Cytophaga-Flavobacterium*	28	29.1 ± 3.8

[a] A formamide concentration of 55% was used in the hybridization buffer for all the probes. Hybridization and washing were performed at 35 to 37°C except for probe Nso190 (hybridization at 46°C and washing at 48°C). The concentration of sodium chloride in the washing buffer was 10 mM except for probe Nso190 (20 mM).
[b] Used with an equimolar amount of unlabeled competitor oligonucleotides.
[c] Means ± standard deviations for triplicate determinations.

This illustrates that a combination of CARD-FISH and flow sorting might be a powerful approach to study the diversity and potentially the activity and the genomes of different bacterial populations in aquatic habitats.

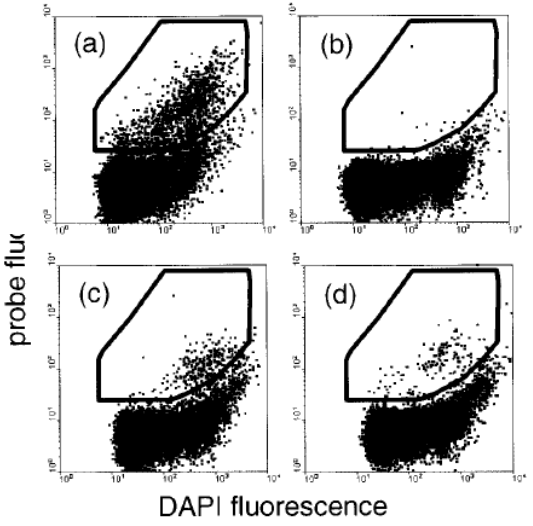
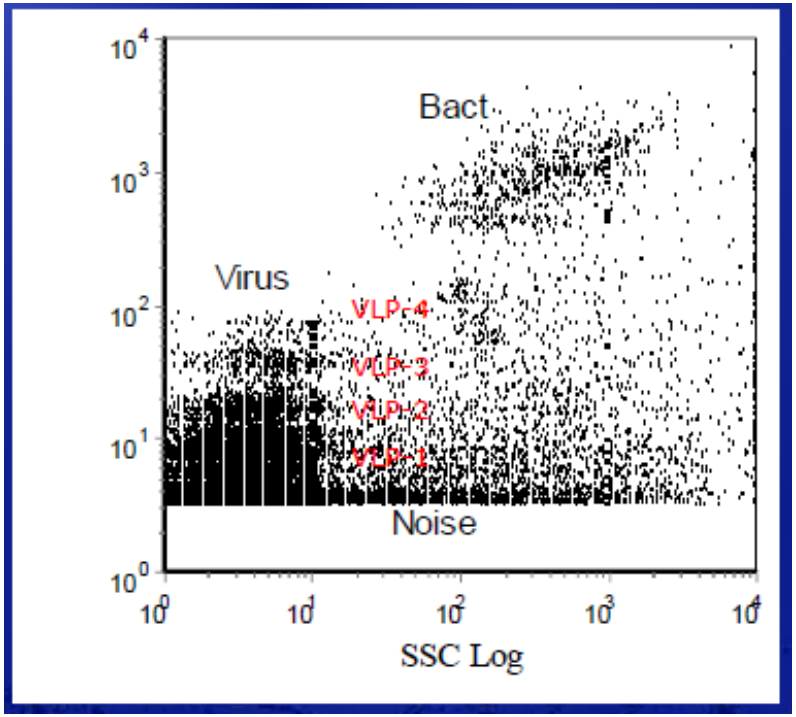


FIG. 3. Flow cytometric analysis of bacterial cells in North Sea surface water sample after CARD-FISH and resuspension. (a) Probe EUB338. (b) Probe NON338. (c) Probe CF319a. (d) Probe BET42a. The dot plots are plots of DAPI fluorescence versus probe fluorescence. Double-stained cells within the gate indicated were sorted for subsequent molecular analysis.

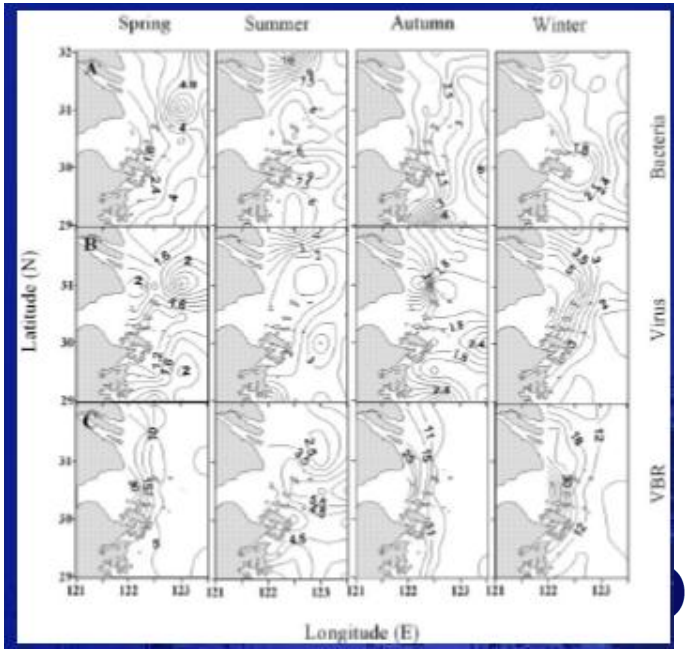
水样中病毒检测

- 标记病毒DNA，根据病毒和细菌大小以及DNA含量的不同进行区分；
- 利用荧光染料标记的特异识别病毒抗原的单克隆抗体，可以检测细胞表面及细胞内的病毒抗原。



海洋浮游病毒的流式细胞分析图谱
图片来源：厦门大学

解析了长江口浮游病毒动态变化，发现来自高度人为干预的黄浦江水的扰动是自然规律失常的主要原因。（Jiao *et al*., 2006）



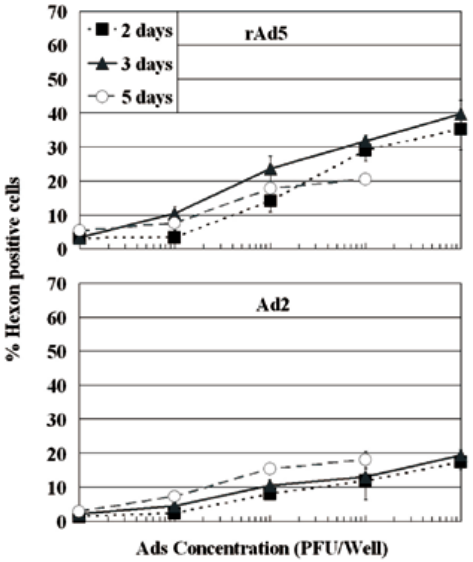
长江口细菌、病毒丰度的季节变化

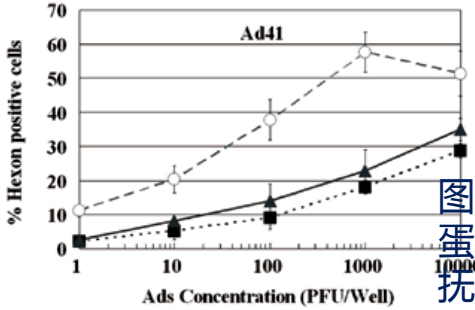
检测细胞表面及细胞内的病毒抗原

Applied and Environmental Microbiology

Detection of Infectious Adenoviruses in Environmental Waters by Fluorescence-Activated Cell Sorting Assay

Dan Li, Miao He and Sunny C. Jiang
Appl. Environ. Microbiol. 2010, 76(5):1442. DOI: 10.1128/AEM.01937-09.
Published Ahead of Print 15 January 2010.





图释：流式细胞仪在HEK-293A细胞上基于腺病毒六邻体蛋白检测rAd5、Ad2和Ad41。图中显示原初病毒密度和抚育时间对于检测的影响。

表中显示利用此法从环境样本中检测感染性腺病毒。

TABLE 1. Detection of infectious adenoviruses in environmental samples by FACS based on viral hexon protein

Sampling source[a]	Sampling date[b]	Sample type	No. positive by FACS	Hexon-positive cells (%)	Estimated Ads in water samples (PFU/100 ml)
Coastal Pacific, Los Angeles	02/26/09–02/13/09	Seawater	0 of 10		
S. Cal. sewage treatment facility	03/31/09–04/08/09	Primary effluents	4 of 6	5 to 8	10–165
S. Cal. sewage treatment facility	03/31/09–04/08/09	Secondary effluents	4 of 12	4 to 6	10–105

[a] S. Cal., southern California.
[b] Month/day/year.

流式在水体微型生物研究中的应用

- 病毒和细菌的检测
  - 计数
  - 活力检测
  - 分类鉴定
- 藻类
  - 水体藻类的分类和多样性研究 WHO ?
  - 水体藻类的活性和功能检测 WHAT ?
  - 水体藻类分选应用 WHY ?

水体藻类的分类和多样性研究

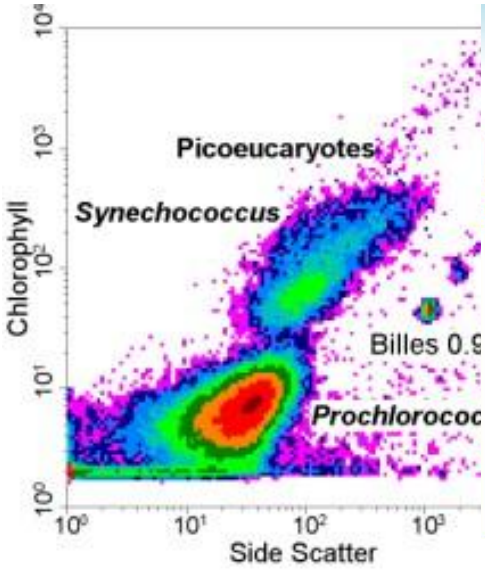
水生浮游植物种类多、数量大、分布广，是生态系统中不可或缺的一环。

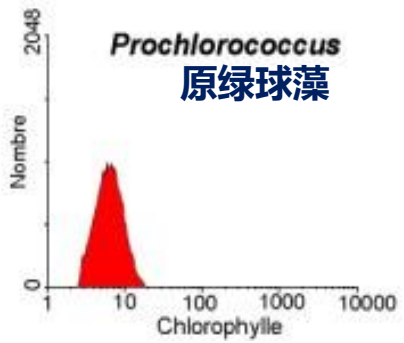
水生浮游植物的群落、数量及分布信息对于了解淡水生态系统动力学具有重要意义。

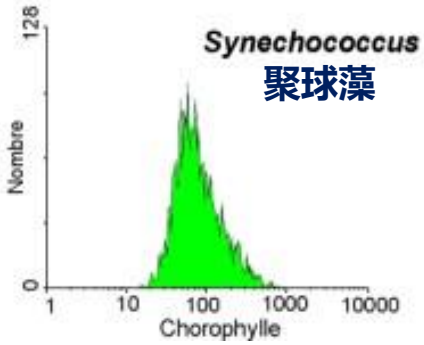
对淡水生态系统保护、资源的开发利用提供理论支持。

流式细胞术可以根据浮游植物固有性质(细胞大小和颗粒度，色素自发荧光) 或结合荧光染料，对其进行分群，并同步研究个类群的数量和生理生化变化。

根据藻类自发荧光定义水体中藻类







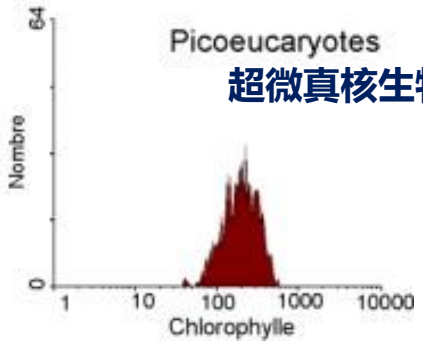


Image provided by Daniel Vaulot,CNRS, Station Biologique de Roscoff, France

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应用举例：藻类群落分析

Author version: *J. Exp. Mar. Biol. Ecol., vol.426-427; 2012; 88-96*

Picophytoplankton community from tropical marine biofilms

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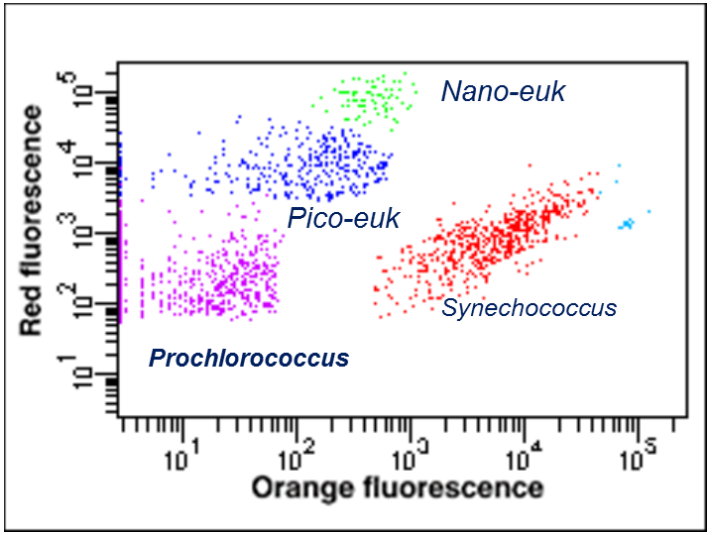
Council of Scientific and Industrial Research, National Institute of Oceanography, Dona Paula - 403 004, Goa, India

2.3. Flow cytometric analysis

A BD FACSAria™ II flow cytometer equipped with a laser emitting at 488 nm and a 70 µm nozzle was used for picoplankton analysis. Emitted light was collected through the following set of filters: 488/10 band pass (BP) for side scatter, 575/26 BP for orange fluorescence, 530/30 BP for green fluorescence and 695/40 BP for red fluorescence. Picophytoplankton groups could be discriminated and enumerated according to their specific autofluorescence properties and light scatter differences (Fig. 1). For the heterotrophic bacterial counts, samples were quickly thawed and incubated in the dark for 30 min at 37^{0}C after addition of SYBR–Green I (final concentration 10^{-4}; Molecular probes) which was used as the nucleic acid stain (Marie et al., 1997). Fluorescent beads (2 µm for picophytoplankton and 1µm for bacteria; "Fluoresbrite", polysciences) were used as internal standards and for calibration of the above parameters. Flow cytometric data were collected and saved as listmode files.



应用举例：分析群落空间和时间的变化规律

Phytoplankton population dynamics at the Bermuda Atlantic Time-series station in the Sargasso Sea

Michele D. DuRand[a,b,*], Robert J. Olson[a], Sallie W. Chisholm[c]

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Abstract

Phytoplankton populations were analyzed using flow cytometry in monthly samples at the Bermuda Atlantic Time-series Study (BATS) station in the Sargasso Sea from 1989–1994 for picoplankton (*Synechococcus* and *Prochlorococcus*) and from 1992–1994 for eukaryotic phytoplankton in order to better understand the mechanisms that dictate seasonal and inter-annual patterns in the phytoplankton community. The eukaryotic phytoplankton were dominated by populations of small nanoplankton (mostly 2–4 μm diameter), though populations of coccolithophores and sometimes pennate diatoms also could be distinguished. Flow cytometric measurements of population abundances, individual cell light scattering (which can be related to cell size), and chlorophyll fluorescence were made. *Synechococcus* and the eukaryotic phytoplankton reached their greatest concentrations during the spring bloom each year when the water column was deeply mixed and nutrients were detectable in surface waters. The maximum cell concentration for *Prochlorococcus* was in the summer and fall of each year, with a deeper sub-surface maximum than *Synechococcus*. Picoplankton chlorophyll fluorescence and estimated cell size were greater at depth than near the surface, and were lowest in midsummer for both *Synechococcus* and *Prochlorococcus*. In the summer and fall, *Prochlorococcus* cells were often smallest at mid-depth, even when fluorescence per cell and cell concentration were lower at the surface. For the eukaryotes (including coccolithophores), cell concentrations were high during the spring in both 1992 and 1993, and in fall 1992. At these times, mean cell size and fluorescence were low. Improved size and carbon estimates were made and it was found that the estimated contribution of phytoplankton carbon to total particulate organic carbon, integrated over the upper 200 m, averaged 33% (range 21–43%) with no pronounced seasonal pattern.

利用流式细胞仪调查了百慕大大西洋的浮游藻类的群落结构，分析了浮游植物的季节变化规律，结果表明：聚球藻以及真核浮游植物在春季的时候浓度最高，且在表层浓度达到最高值；而原绿球藻在夏季和秋季的时候浓度最高，在次表层其浓度达到最高值。

应用举例：原绿球藻和聚球藻的世界分布格局

PNAS

Present and future global distributions of the marine Cyanobacteria *Prochlorococcus* and *Synechococcus*

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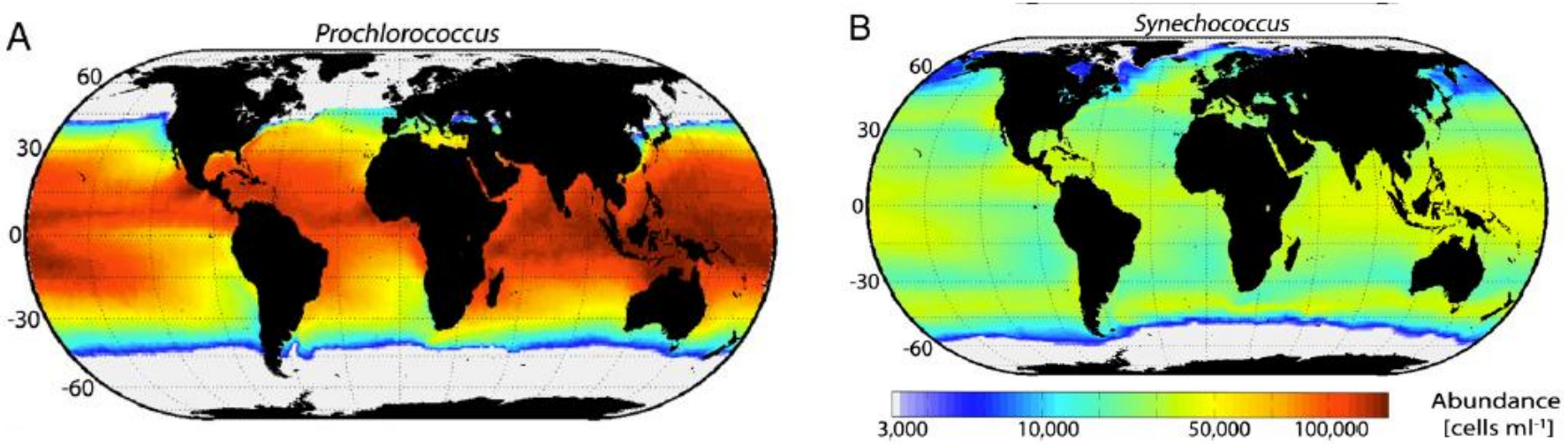
Contributed by David M. Karl, April 25, 2013 (sent for review January 22, 2013)



Present global distribution of Prochlorococcus and Synechococcus abundance. (A) Prochlorococcus and (B) Synechococcus mean annual abundances at the sea surface.

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流式在水体微型生物研究中的应用

- 病毒和细菌的检测
  - 计数
  - 活力检测
  - 分类鉴定
- 藻类
  - 水体藻类的分类和多样性研究 — WHO？
  - 水体藻类的活性和功能检测 — WHAT？
  - 水体藻类分选应用 — WHY？

WHAT？
- 藻类计数
- 藻类活性检测
- 藻类细胞周期检测
- 藻类代谢活性

1. 藻类计数

在海洋浮游植物研究中，光学显微镜技术目前仍然是主要手段，但是对于个体较小的微型藻类及超微型藻类的研究，该手段具有局限性。

人工镜检计数

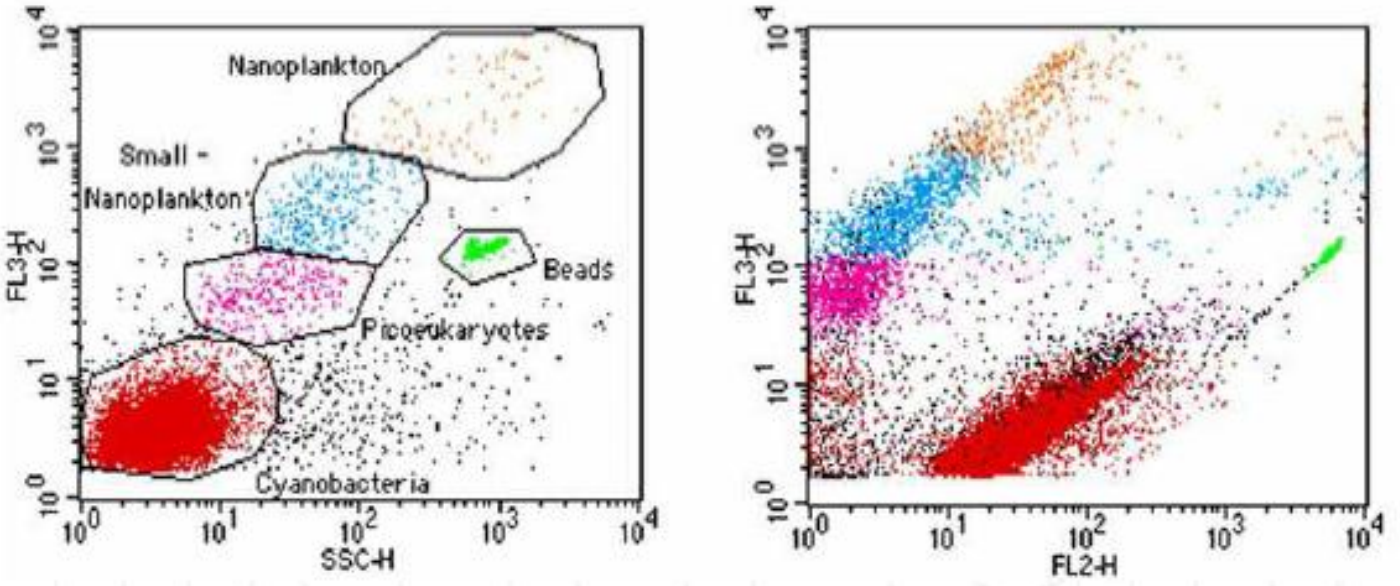
传统方法
费时费力
误差较大

流式细胞仪

无需前处理
节约时间
精确计数

藻类计数

- 绝对计数：
  A. 通过流式细胞仪直接计算出上样体积；
  B. 根据已知浓度的计数微球进行计算；



Number of cells = (cell events/beads events)*
(bead number/µl)* Dilution Factor

应用举例

- 应用于水环境污染的监测：
  - A. 直接从环境中取样，不需要细胞培养和富集；
  - B. 可以准确地分辨出活细胞、死细胞以及干扰的颗粒状物质；
  - C. 判断污染物对浮游藻类的生长活动的影响来，作为判断水体的污染程度的指标；
  - D. 作为环境毒理实验的生态学指标；

应用举例：研究重金属对微藻数量的影响

- 利用流式细胞仪，通过藻类自发荧光和侧向角信号，对藻类计数,绘制出微藻生长抑制曲线，分析重金属离子铜和铅对5种海洋微藻的生长抑制。

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Toxicity and bioaccumulation of copper and lead in five marine microalgae

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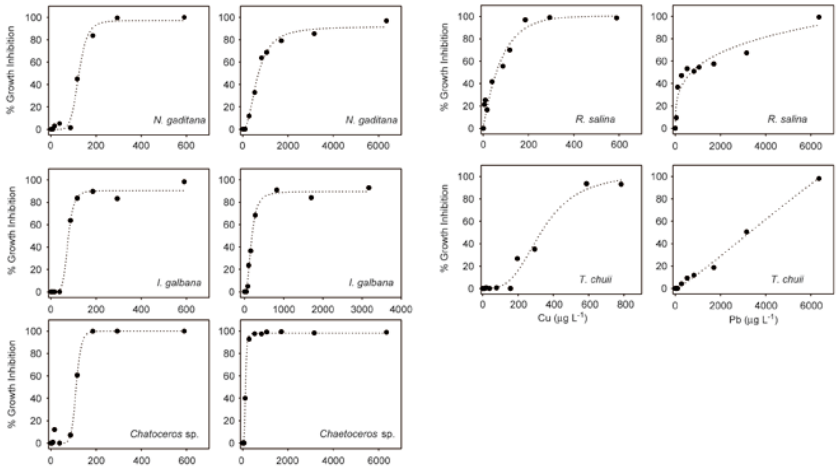
ABSTRACT

On five marine microalgae with the same biovolume quantity (*Tetraselmis chuii*, *Rhodomonas salina*, *Chaetoceros* sp., *Isochrysis galbana* (T-iso) and *Nannochloropsis gaditana*) 72-h exposure toxicity tests with copper and lead were performed. For both metals, 72-h EC50s showed *T. chuii* as the most tolerant and *R. salina* as one of the most sensitive. Besides copper and lead EC_{50} concentrations, metal concentrations in solution and accumulated on/in the cell where also analysed. *T. chuii*, the most tolerant species accumulated high copper concentrations ($EC_{50(Cu)} = 330\,\mu g\,L^{-1}$; $EC_{50(Pb)} = 2600\,\mu g\,L^{-1}$), and *R. salina* the most sensitive to copper, accumulated the highest amount of this metal ($EC_{50(Cu)} = 50\,\mu g\,L^{-1}$). Results of this study show that there is no specific relationship between cell tolerance and accumulated metal on/in the cell. On the other hand, due to an established evidence of the influence of cellular density in microalgae toxicity tests, this effect was also studied. Results showed reduced EC_{50} values when initial cellular densities decreased.

In this study, the term "toxic cellular quota" was used to express all data. This allowed, in a single expression, the combination of two parameters that clearly influence growth, cellular density and toxic concentration.

2.5. Flow cytometry analysis

Samples for analysis by flow cytometry were collected from the toxicity tests after 72 h of treatment. These were analysed using a FACScalibur flow cytometer equipped with a 488 nm excitation argon laser and the data were computed with CellQuest software (Beckton-Dickinson). Each culture was immediately analysed for 30–60 s (6000–10,000 events per measurement) from samples previously fixed with 3–4% formaldehyde. Counts, signals of side-angle light scatter (SSC), and autofluorescence (FL3, >630 nm) were recorded and used as indicators of the cellular size and chlorophyll fluorescence, respectively (Sobrino et al., 2004).



2. 藻类细胞活性检测

Vol. 26: 157–166, 2001 | AQUATIC MICROBIAL ECOLOGY Aquat Microb Ecol | Published December 5

Flow cytometric analysis of phytoplankton viability following viral infection

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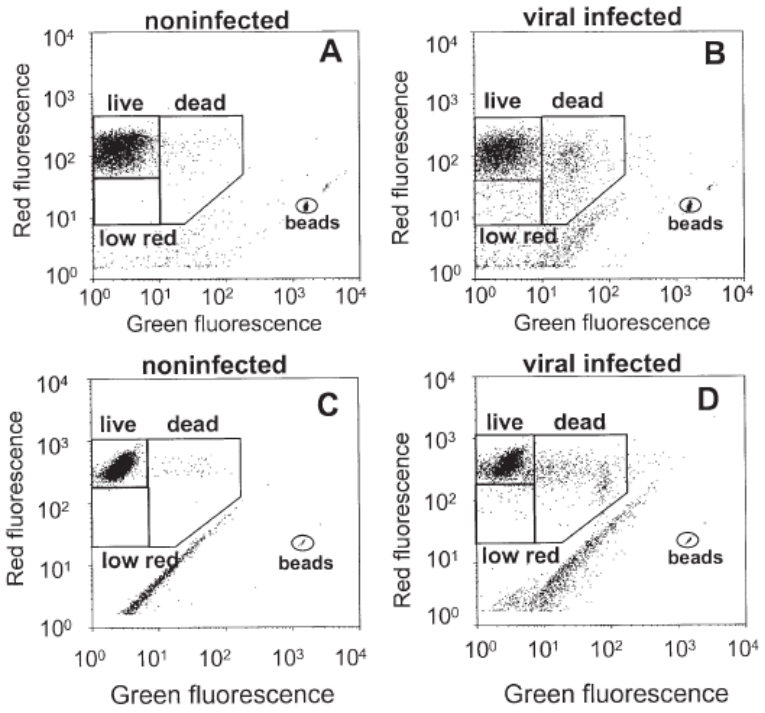
[2]Station Biologique, CNRS, INSU et Université Pierre et Marie Curie, BP 74, 29682 Roscoff cedex, France

Flow cytometry. A FACSort and a FACSCalibur flow cytometer (Becton Dickinson), both equipped with an air-cooled argon laser (excitation 488 nm, 15 mW power) with the standard filter set-up, were used. Analyses were triggered on red autofluorescence and run for 1 to 4 min at a delivery rate of 50 µl min^{-1}. To avoid coincidence, algal cells were enumerated using an event rate between 100 and 400 cells s^{-1}. When needed, algal samples were diluted in 0.2 µm pore size filtered seawater. Sheath fluid for both assays consisted of 0.2 µm pore size filtered seawater.

目的：利用流式细胞仪分析病毒感染对藻类细胞活性影响。
荧光染料：SYTOX-Green （死细胞染料）
激光器：488nm
检测通道：Green fluorescence



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3. 藻类细胞周期检测

- 细胞周期反映了生物自身的代谢和增殖能力，通过细胞周期分析G1期，S期和G2/M期可以深入了解微生物的生长代谢情况。

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1997, p. 186–193
0099-2240/97/$04.00+0

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Enumeration and Cell Cycle Analysis of Natural Populations of Marine Picoplankton by Flow Cytometry Using the Nucleic Acid Stain SYBR Green I

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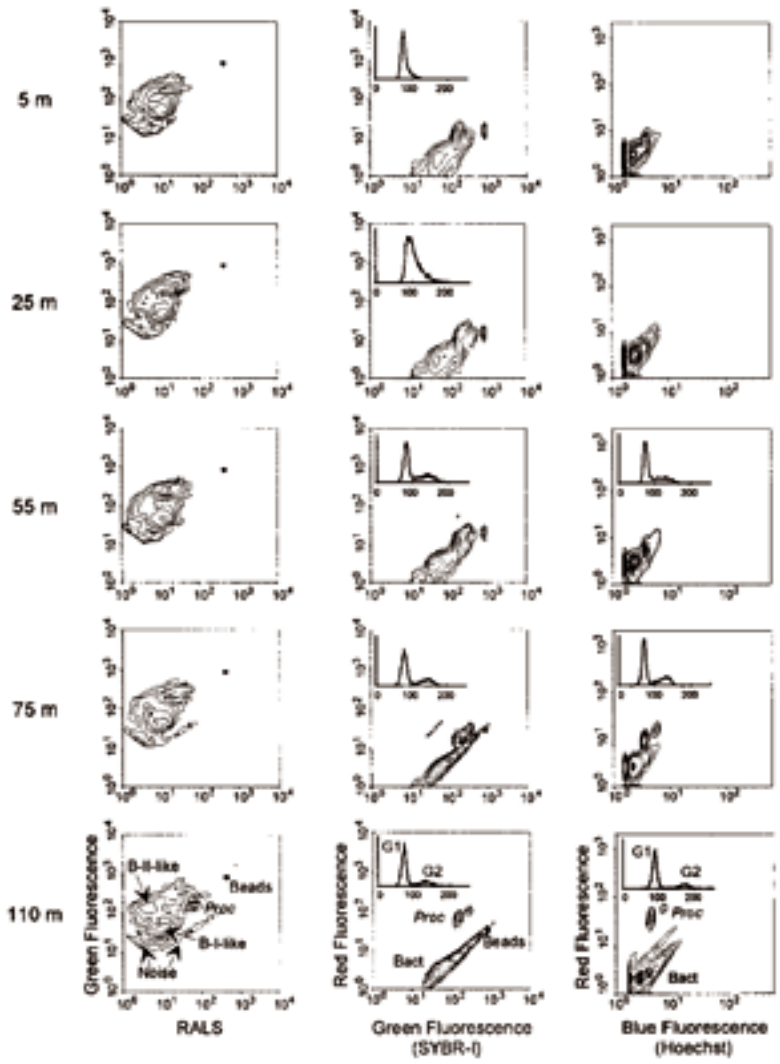
The novel dye SYBR Green I binds specifically to nucleic acids and can be excited by blue light (488-nm wavelength). Cell concentrations of prokaryotes measured in marine samples with this dye on a low-cost compact flow cytometer are comparable to those obtained with the UV-excited stain Hoechst 33342 (bis-benzimide) on an expensive flow cytometer with a water-cooled laser. In contrast to TOTO-1 and TO-PRO-1, SYBR Green I has the advantage of clearly discriminating both heterotrophic bacteria and autotrophic *Prochlorococcus* cells, even in oligotrophic waters. As with TOTO-1 and TO-PRO-1, two groups of heterotrophic bacteria (B-I and B-II-like types) can be distinguished. Moreover, the resolution of DNA distribution obtained with SYBR Green I is similar to that obtained with Hoechst 33342 and permits the analysis of the cell cycle of photosynthetic prokaryotes over the whole water column.

藻类细胞周期检测



流式细胞术分析采集自不同深度的海水中原绿球藻群落细胞周期，样本经过SYBR-I(左、中列)或Hoechst 33342(右列)染色处理。

TABLE 2. Percentages of cells in the different phases of the cell cycle and CVs of the G_1 peak for natural *Prochlorococcus* populations[a]

Depth (m)	SYBR-I Green				Hoechst 33342			
	CV G_1 (%)	%G_1	%S	%G_2	CV G_1 (%)	%G_1	%S	%G_2
5	9.0	88.3	11.7	0.0				
15	9.0	80.7	19.3	0.0				
25	12.0	69.0	31.0	0.0				
35	7.6	21.4	78.6	0.0				
45	7.1	38.4	56.6	4.9				
55	8.6	58.8	29.1	12.1	7.2	59.5	33.0	7.6
65	8.2	77.6	21.2	1.2	7.5	74.5	24.1	1.4
75	8.8	65.5	12.8	21.6	7.8	63.2	9.0	21.5
85	9.5	56.6	10.7	32.6	7.4	55.3	9.9	31.4
95	9.8	71.5	4.5	24.1	7.2	69.5	8.4	22.0
110	8.8	75.8	7.8	16.4	7.2	78.8	7.5	14.8

[a] Data shown are for natural *Prochlorococcus* populations from the equatorial Pacific Ocean (OLIPAC cruise, cast number 73) stained with either Hoechst 33342 or SYBR-I. The data were processed with the Multicycle software.

4. 藻类代谢活性

检测藻的脂酶活性用FDA（ Fluorescein Diacetate ）染色，代谢探针，激发波长488nm，在FLI通道处收集FDA荧光，荧光检测波长为530—560nm

F. J. Jochem

Dark survival strategies in marine phytoplankton assessed by cytometric measurement of metabolic activity with fluorescein diacetate

Received: 10 May 1999 / Accepted: 20 September 1999

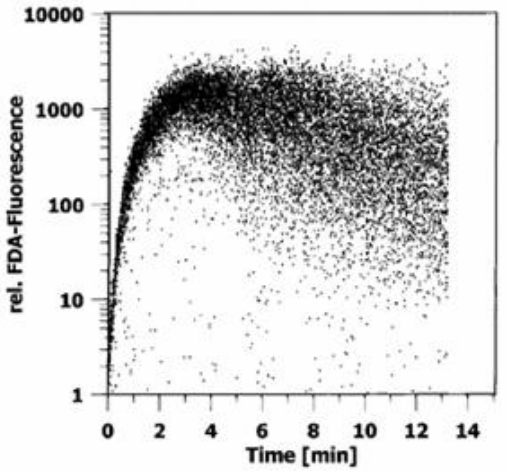


Fig. 1 Time course of fluorescence (relative units) accumulation upon FDA addition in *Brachiomonas submarina* as revealed by flow cytometry

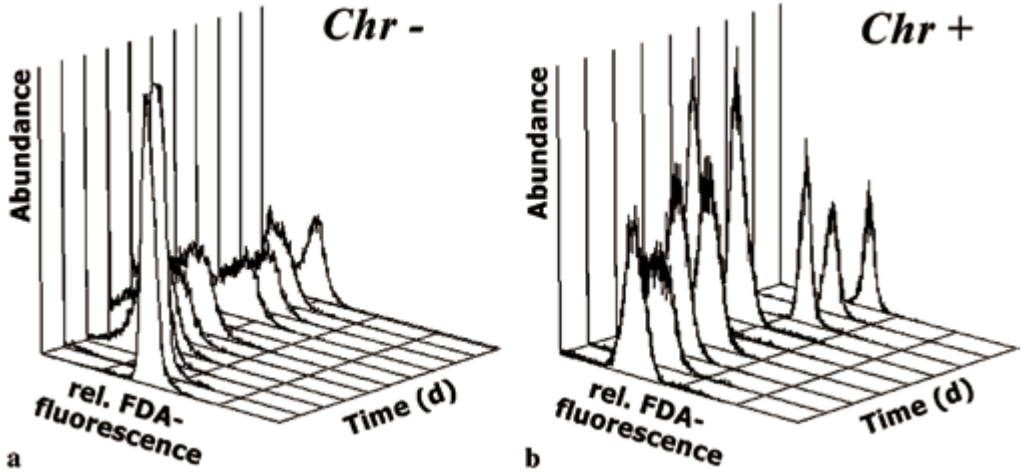


Figure 2 Daily frequency distri- butions of FDA-derived fluo- rescence (relative units) in Chrysochromulina hirta kept in darkness a without (Chr)) and b with (Chr+) addition of bacteria

流式细胞仪分析Chrysochromulina hirta中FDA来源的荧光强度的每日的频率分布。细胞内的酯酶可以催化FDA形成荧光，流式细胞仪通过检测荧光来评价浮游植物的代谢活力。

藻类代谢活性

Development of an Improved Rapid Enzyme Inhibition Bioassay with Marine and Freshwater Microalgae Using Flow Cytometry

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Abstract. A rapid toxicity test based on inhibition of esterase activity in marine and freshwater microalgae (*Selenastrum capricornutum*, *Chlorella* sp., *Dunaliella tertiolecta*, *Phaeodactylum tricornutum*, *Tetraselmis* sp., *Entomoneis* cf. *punctulata*, *Nitzschia* cf. *paleacea*) was developed using flow cytometry. Uptake of fluorescein diacetate (FDA) was optimized for each species by varying the substrate concentration, incubation time, and media pH. Propidium iodide (PI) was utilized to assess membrane integrity. The optimized FDA/PI staining procedure was then used to assess the toxicity of copper in short-term exposures (1–24 h). Esterase activity was a sensitive indicator of copper toxicity in *S. capricornutum* and *E.* cf. *punctulata*. As copper concentrations increased, esterase activity decreased in a concentration-dependent manner. The 3- and 24-h EC_{50} values (based on mean activity states) were 112 µg Cu L^{-1} (95% confidence limits 88–143) and 51 µg Cu L^{-1} (95% confidence limits 38–70) for *S. capricornutum* and 47 µg Cu L^{-1} (95% confidence limits 43–51) and 9.1 µg Cu L^{-1} (95% confidence limits 7.6–11) for *E.* cf. *punctulata*, respectively. This enzyme inhibition endpoint showed similar sensi-

cells divide daily over this pe
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Davies 2000). Algal metaboli
a drift in pH (Nyholm and K
chemical alteration of the test
tion that detect acute, sublethal
enzyme activity, show promi
tions.

Enzyme inhibition measure
oxidases, β-galactosidases, es
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Peterson and Stauber 1996; Bl
activity, in particular, has pro
types (Berglund and Eversma
1992; Humphreys *et al.* 1994;
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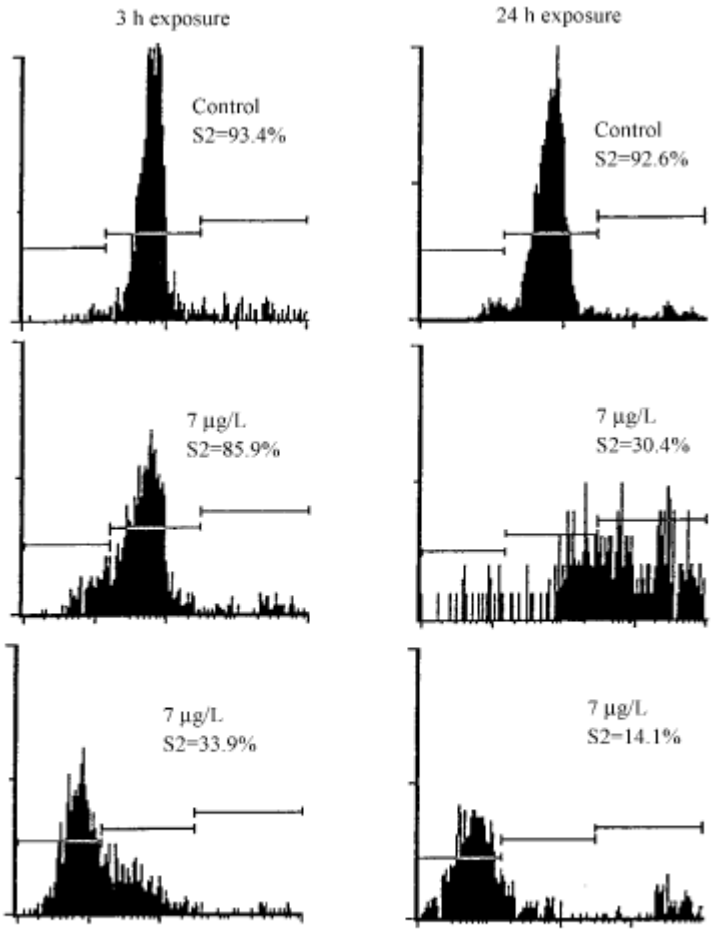


图 1　不同浓度 Cu 离子(0, 7, 70 µg L^{-1})作用 *Selenastrum capricornutum* 3 h 和 24 h 的脂酶活性变化频率直方图

Fig. 1　Flow cytometric histogram showing shifts in esterase activity of *S. capricornutum* at Cu concentrations of 0, 7 and 70 µg L^{-1} after 3 h and 24 h exposure

流式检测不同Cu离子对淡水绿藻脂酶活性的影响。

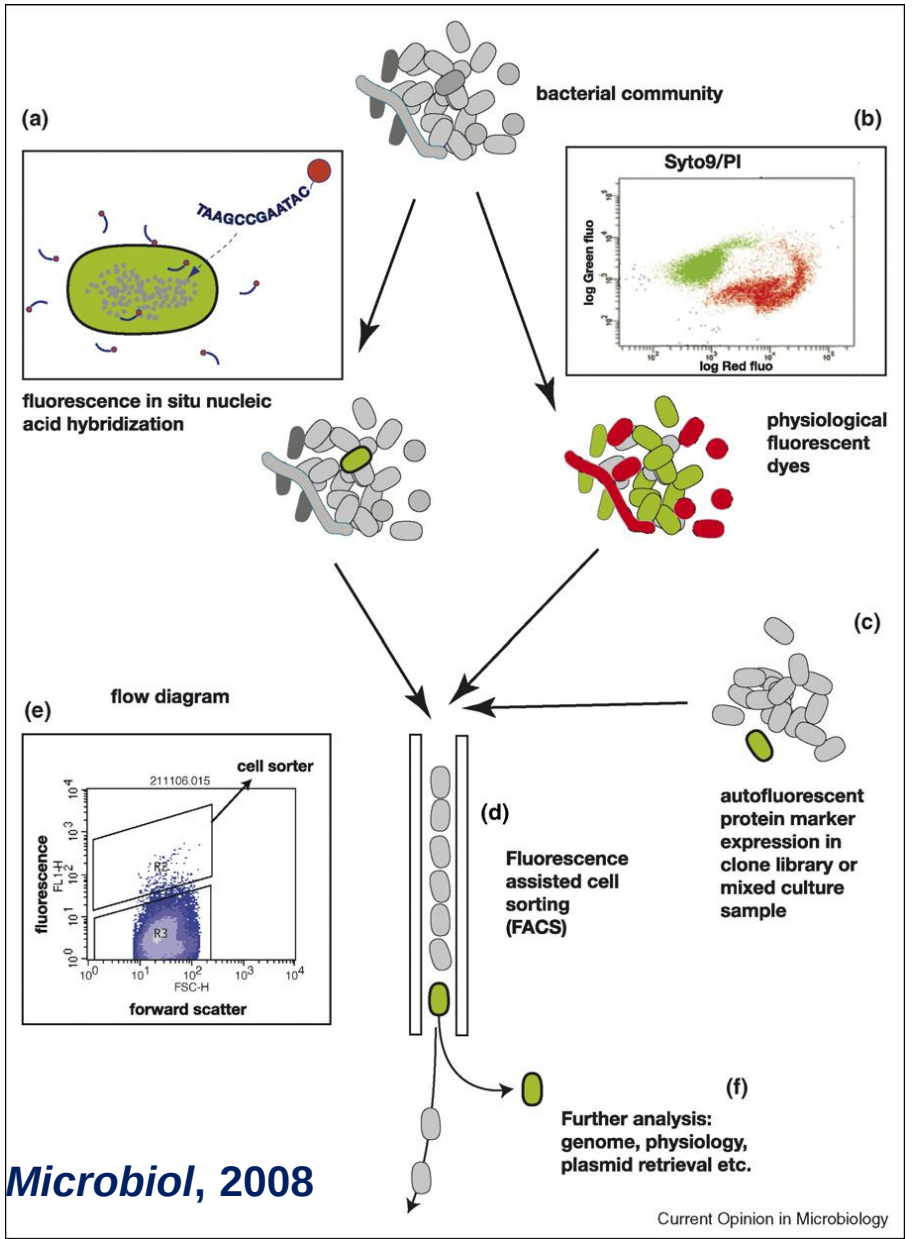
流式在水体微型生物研究中的应用

- 病毒和细菌的检测
  - 计数
  - 活力检测
  - 分类鉴定
- 藻类
  - 水体藻类的分类和多样性研究 WHO ?
  - 水体藻类的活性和功能检测 WHAT ?
  - 水体藻类分选应用 WHY ?

藻类分选应用

流式细胞仪可以通过浮游生物***固有属性***(色素自发荧光、细胞大小和颗粒性)或***特异性荧光探针***对混合样品中的特定组分进行分选，分选获得的细胞可以用于：

- 纯化功能类群
- 生理生化分析
- 分子生物学检测



Czechowska *et al*., *Curr Opin Microbiol*, 2008

应用举例：纯化功能类群

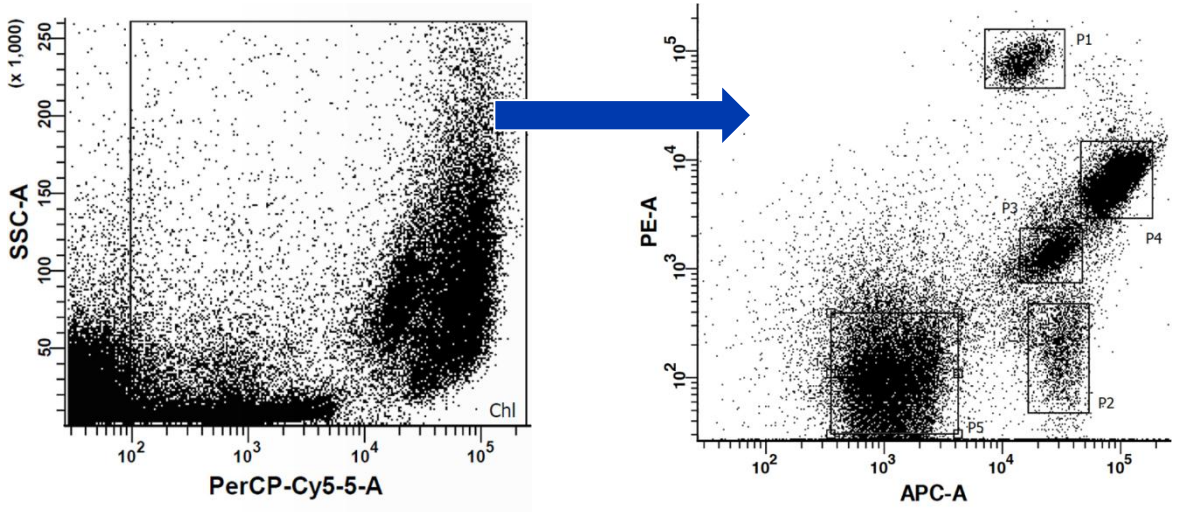
Pereira *et al. Biotechnology for Biofuels* 2011, 4:61
http://www.biotechnologyforbiofuels.com/content/4/1/61

Biotechnology for Biofuels

METHODOLOGY Open Access

Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae

Hugo Pereira[1], Luísa Barreira[1], André Mozes[2], Cláudia Florindo[2], Cristina Polo[1], Catarina V Duarte[1], Luísa Custódio[1] and João Varela[1*]



FACS

The flow cytometer used in our studies was a Becton Dickinson FACS Aria II (BD Biosciences, Erembodegem, Belgium). Fluorescence readings were performed by excitation with a blue and red laser (488 and 633 nm, respectively). The emission signal was measured in three channels upon excitation with the blue laser: FL1 channel centered at 530/30 nm; FL2 centered at 585/42 nm; and FL3 centered at 695/40 nm. A fourth channel, FL4, registered the emission at 660/20 nm after excitation with the red laser.

Samples were acquired with the software FACSDiva version 6.1.3 (BD Biosciences, Erembodegem, Belgium). After the acquisition of samples, images were treated with the analysis software, Infinicyt 1.5.0 (Cytognos S.L., Santa Marta de Tormes, Spain).

The settings and compensations of all channels and lasers were the same for all sorting procedures. The flow cytometry sheath fluid used in all experiments was sterile filtered seawater. Filters (PALL) used had a pore size of 0.2 μm. Sorting was performed at 2,000 events/s flow rate using 'single cell' sort precision mode, with a 100 μm nozzle.

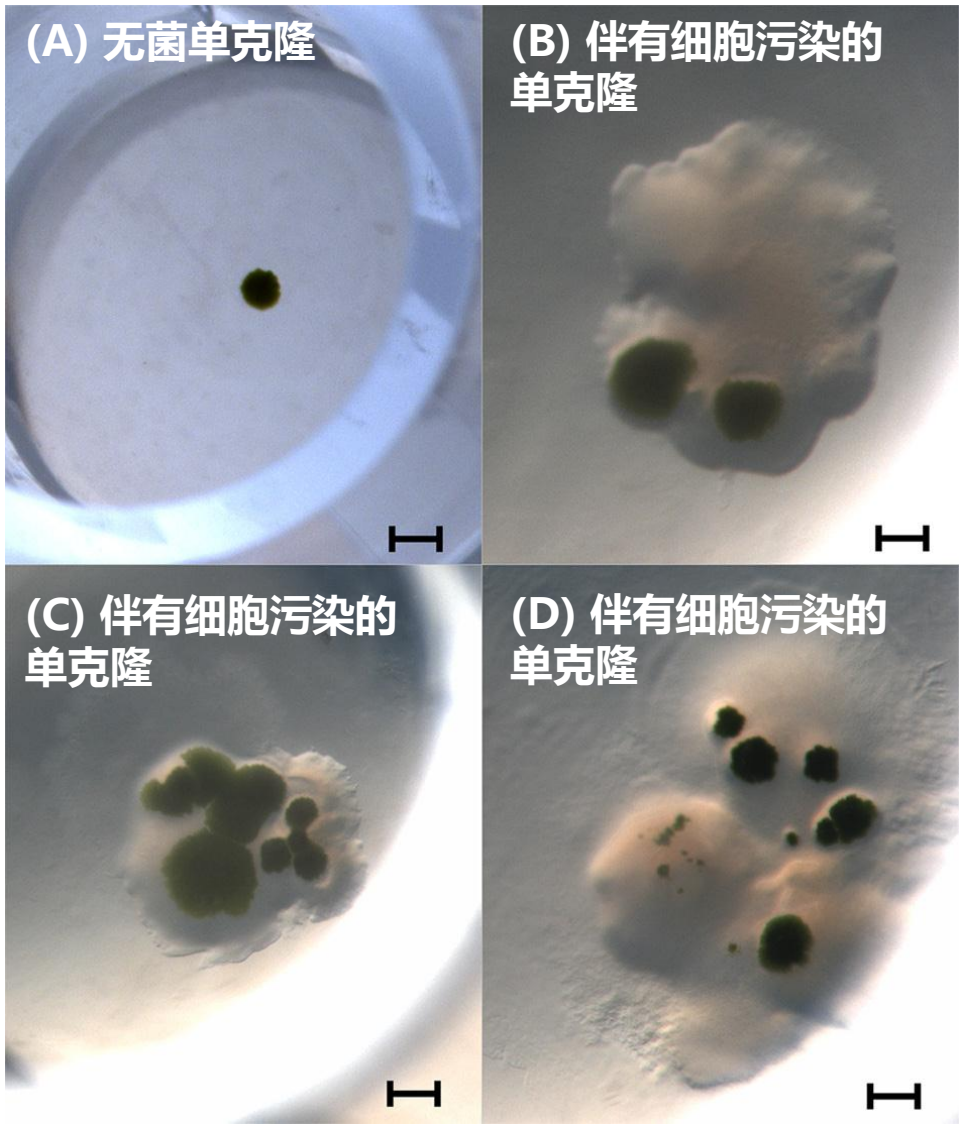
Cells were sorted directly into wells of 96-well plates containing 250 μl of either liquid or solid (agar) Algal growth medium. In order to assess the best number of cells needed to achieve visible culture growth in a feasible

根据水体中微藻类自发荧光（APC通道为藻青蛋白荧光，PE通道为藻红蛋白荧光），将藻类分成5群，并进行分选。

BD

Helping all people live healthy lives

应用举例：纯化功能类群



- 通过分选型流式细胞仪，将细胞分选于96孔板中，分选的细胞可以在含有固体琼脂培养基的96孔板中生长。
- 实验证明，流式细胞分选技术可以进行复杂环境样本的分群以及无菌单细胞克隆的制备。

应用举例：高脂含量微藻分选

Isolation of high-lipid content strains of the marine microalga *Tetraselmis suecica* for biodiesel production by flow cytometry and single-cell sorting

María F. Montero · Manuela Aristizábal · Guillermo García Reina

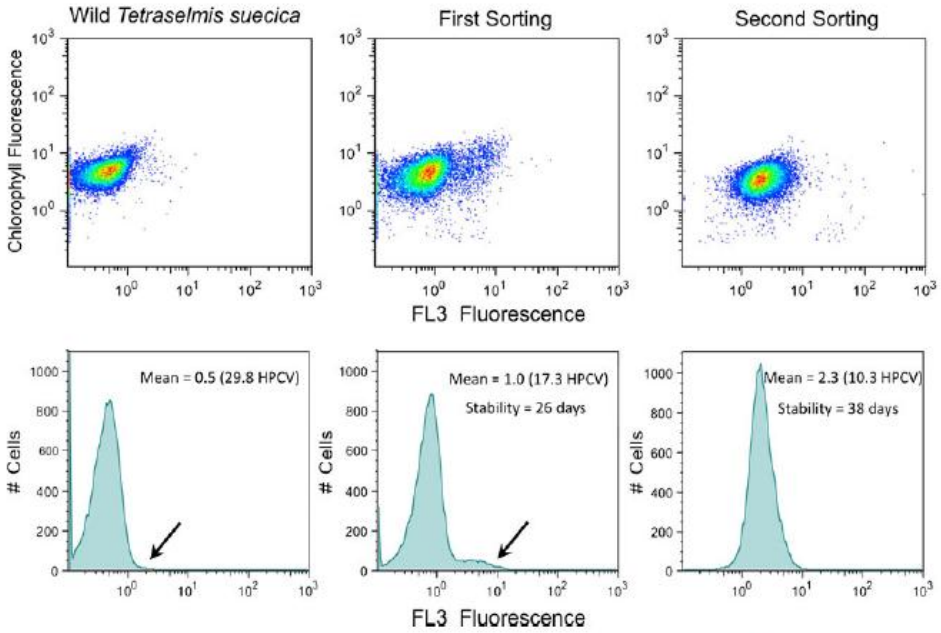


Fig. 3 Isolation and sorting of high-lipidic strains of *T. suecica* during two consecutive steps. The first sorting (8% of the population) was obtained from a wild population (15–20×10^6 cells mL^{-1}) cultured in a photobioreactor under greenhouse sunlight. After 26 days from the first sorting event, the algae (1.2×10^6 cells mL^{-1}) yielded a mean FL3 of 1.0 (17.3 HPCV). The second sorting, 38 days after, yielded a mean FL3 of 2.3 (10.3 HPCV). The *arrows* indicate the sorting regions

图释：利用流式细胞术分析高脂含量T. suecica细胞群，并加以两次分选。第一次分选从生长于光合生物反应器的15–20×10^{6}cells mL^{-1}细胞中获得了8%的细胞群。26天后，1.2×10^{6}cells mL^{-1}细胞可以产生的平均 FL3为1。第二次分选38天后，可以产生的平均 FL3为2.3。细胞经过Nile Red染色处理，该染料为一种亲脂性染料。

应用举例：不同群体细胞的生理生化分析

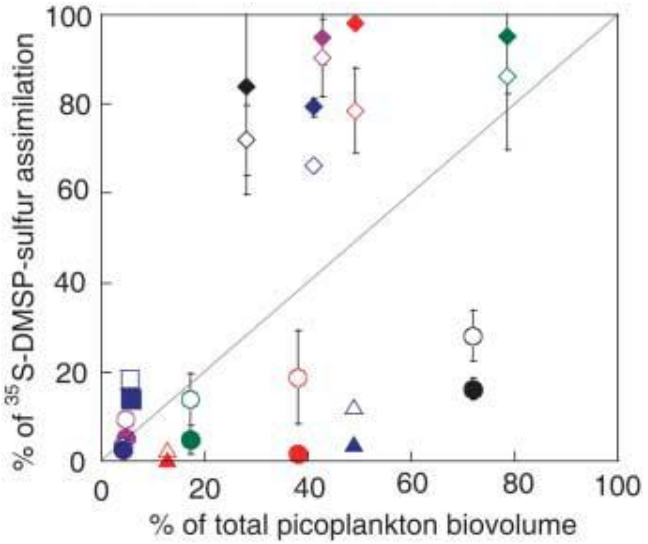
Science
AAAS

Dimethylsulfoniopropionate Uptake by Marine Phytoplankton
Maria Vila-Costa, *et al.*
Science **314**, 652 (2006);
DOI: 10.1126/science.1131043

Dimethylsulfoniopropionate Uptake by Marine Phytoplankton

Maria Vila-Costa,[1]* Rafel Simó,[1]* Hyakubun Harada,[2] Josep M. Gasol,[1]
Doris Slezak,[2] Ronald P. Kiene[2]

Dimethylsulfoniopropionate (DMSP) accounts for most of the organic sulfur fluxes from primary to secondary producers in marine microbial food webs. Incubations of natural communities and axenic cultures with radio-labeled DMSP showed that dominant phytoplankton groups of the ocean, the unicellular cyanobacteria *Prochlorococcus* and *Synechococcus* and diatoms, as well as heterotrophic bacteria take up and assimilate DMSP sulfur, thus diverting a proportion of plankton-produced organic sulfur from emission into the atmosphere.



- 利用流式细胞仪FACSCalibur-Cell Sorter分析并分选超微浮游植物中不同组分的DMSP硫摄取和同化的分布。
- 流式细胞术将样本中的浮游生物分为四个主要类群：异样细菌、原绿球藻、集球藻属和自发体荧光超微型真核生物。所有类群均表现出一定的同化DMSP中硫的能力。

分选应用：分子生物学检测

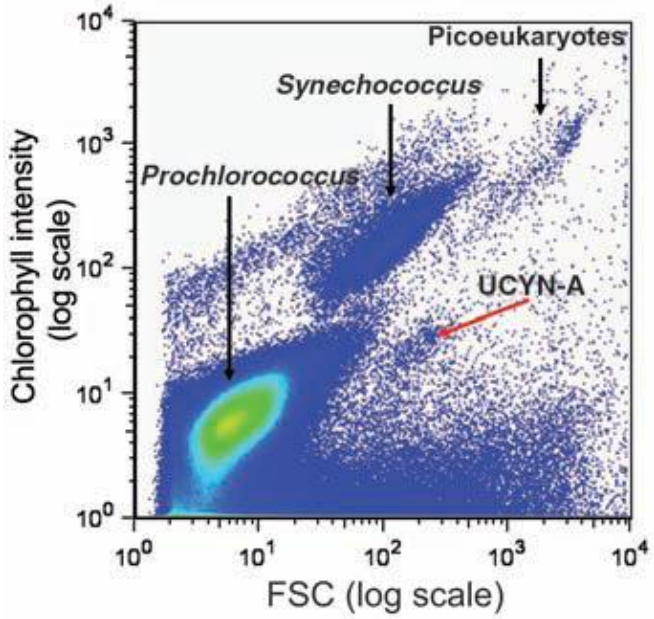
Science
AAAS

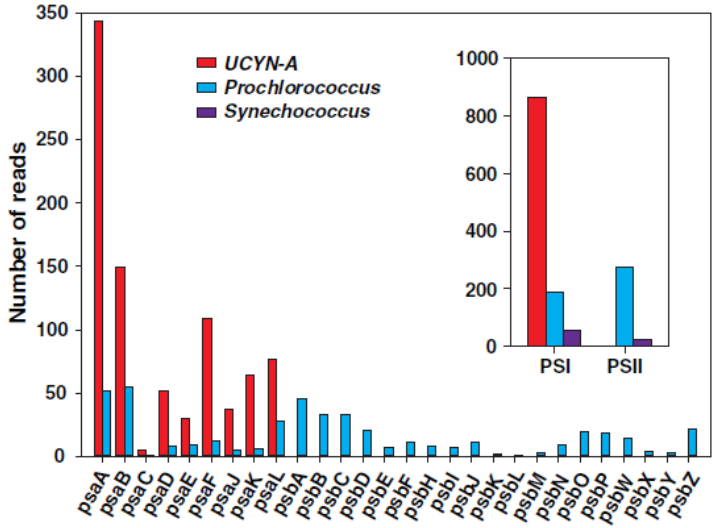
Globally Distributed Uncultivated Oceanic N2-Fixing Cyanobacteria Lack Oxygenic Photosystem II
Jonathan P. Zehr, *et al.*
Science **322**, 1110 (2008);
DOI: 10.1126/science.1165340

Globally Distributed Uncultivated Oceanic N_2-Fixing Cyanobacteria Lack Oxygenic Photosystem II

Jonathan P. Zehr,[1]* Shellie R. Bench,[1] Brandon J. Carter,[1] Ian Hewson,[1] Faheem Niazi,[2] Tuo Shi,[1] H. James Tripp,[1] Jason P. Affourtit[2]

Biological nitrogen (N_2) fixation is important in controlling biological productivity and carbon flux in the oceans. Unicellular N_2-fixing cyanobacteria have only recently been discovered and are widely distributed in tropical and subtropical seas. Metagenomic analysis of flow cytometry–sorted cells shows that unicellular N_2-fixing cyanobacteria in "group A" (UCYN-A) lack genes for the oxygen-evolving photosystem II and for carbon fixation, which has implications for oceanic carbon and nitrogen cycling and raises questions regarding the evolution of photosynthesis and N_2 fixation on Earth.





利用流式对原绿球藻，聚球藻和单细胞蓝藻进行分选，并分别进行基因组分析，发现固氮蓝藻缺乏放氧光合系统II，因此无法进行固碳作用。

单细胞测序——荣登年度技术榜首

2014年年初，***Nature Methods***将单细胞测序选为年度最重要的方法学进展，对单个细胞基因信息的解读，成为科学研究的新焦点。

EDITORIAL

Method of the Year 2013

Methods to sequence the DNA and RNA of single cells are poised to transform many areas of biology and medicine.

Once considered a technical challenge reserved for a few specialized labs, single-cell transcriptome and genome sequencing is becoming robust and broadly accessible. Exciting insights from recent studies are revealing the potential to understand biology at the unitary resolution of life, and last year marked a turning point in the widespread adoption of these methods to address a variety of research questions. For these reasons, single-cell sequencing is our choice of Method of the Year for 2013.

Every cell is unique—it occupies an exclusive position in space, carries distinct errors in its copied genome and is subject to programmed and induced changes in gene expression. Yet most DNA and RNA sequencing is performed on samples or cell populations, in which differences between cells can be obscured by averaging for technical noise.

high-throughput sequencing. Maintaining fidelity and avoiding biases during heavy amplification is not trivial, but doing so is critical to ensuring adequate sequence coverage, accurate quantification and detection of sequence variation.

Recent protocol improvements and commercial offerings are helping to ease the adoption of single-cell sequencing approaches. Microfluidics and microwell technologies are also improving reproducibility and scale. We outline some basic workflows and considerations in a Primer (p. 18). In a News Feature (p. 13), Kelly Rae Chi highlights how single-cell sequencing approaches are already being effectively applied in the areas of biological development, cancer and neurobiology.

Single-cell genome sequencing reduces the sequence complexity of cell mixtures. In a Commentary (p. 19), Paul Blainey and Stephen Quake discuss how this can be leveraged to determine recombination frequencies in cells undergoing meiosis, to tease apart the maternal and paternal genomic contributions, or haplotypes, and to enable the assembly of microbial genomes sampled directly from complex mixtures in the environment.

In another Commentary, Rickard Sandberg argues that we are entering an era of single-cell transcriptome sequencing that will deepen our understanding of gene regulation and cellular transcriptional states, improve our ability to identify differences between healthy and diseased tissues, and profile rare cancerous cells (p. 22).

By focusing on genome and transcriptome sequencing, we do not mean to discount the importance of alternative single-cell approaches. Other methods such as *in situ* hybridization can effectively interrogate sequences in single cells in addition to providing the physical address of transcripts or DNA in intact tissue. Epigenomic profiling of single cells will add important information on gene regulation. Beyond sequence, approaches such as mass cytometry and mass spectrometry will help to characterize protein expression in single cells on a large scale. A final Commentary by James Eberwine and colleagues (p. 25) discusses the directions that such complementary technologies will need to take to understand single cells at the level of function.

We also present our Methods to Watch (p. 28), a selection of methods or areas of methodological development that we believe have particularly interesting potential in the coming years.

We hope that you enjoy our special feature. A happy 2014 to all our readers!

SPECIAL FEATURE

Method of the Year 2013

Special Feature

- Contents
- Editorial
- News feature
- Primer
- Commentary
- Methods to Watch

Nature Methods' choice for Method of the Year 2013 is single-cell sequencing. A collection of articles present the unique considerations related to sequencing single cells and highlight recent applications in biology and medicine. The Methods to Watch feature provides a look at possible future Methods of the Year.

多细胞步向单细胞研究

European Pharmaceutical Review
Volume 16, Issue 2, 2011
www.europeanpharmaceuticalreview.com/qPCR

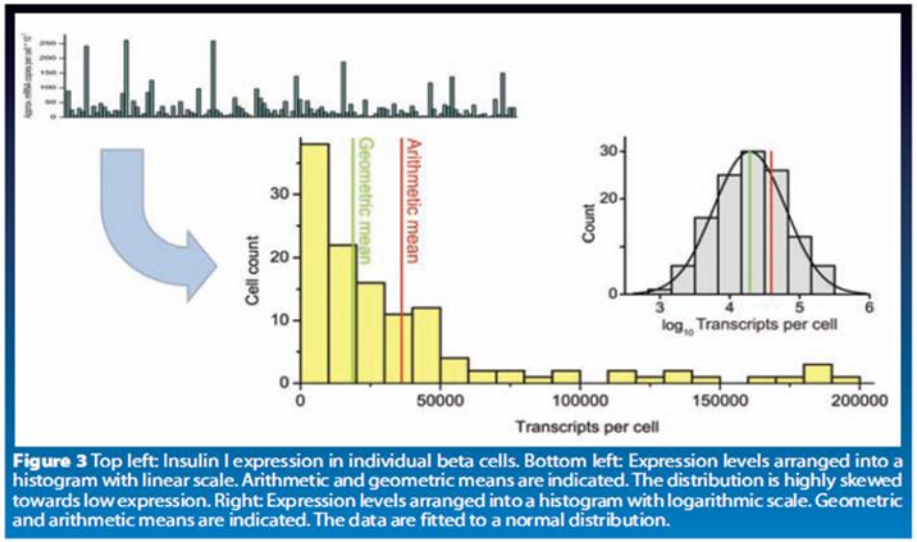


Figure 3 Top left: Insulin I expression in individual beta cells. Bottom left: Expression levels arranged into a histogram with linear scale. Arithmetic and geometric means are indicated. The distribution is highly skewed towards low expression. Right: Expression levels arranged into a histogram with logarithmic scale. Geometric and arithmetic means are indicated. The data are fitted to a normal distribution.

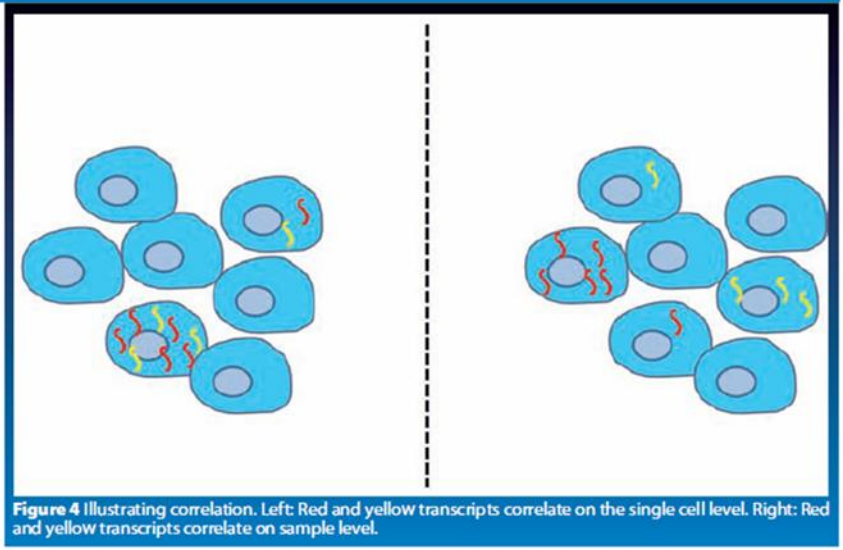
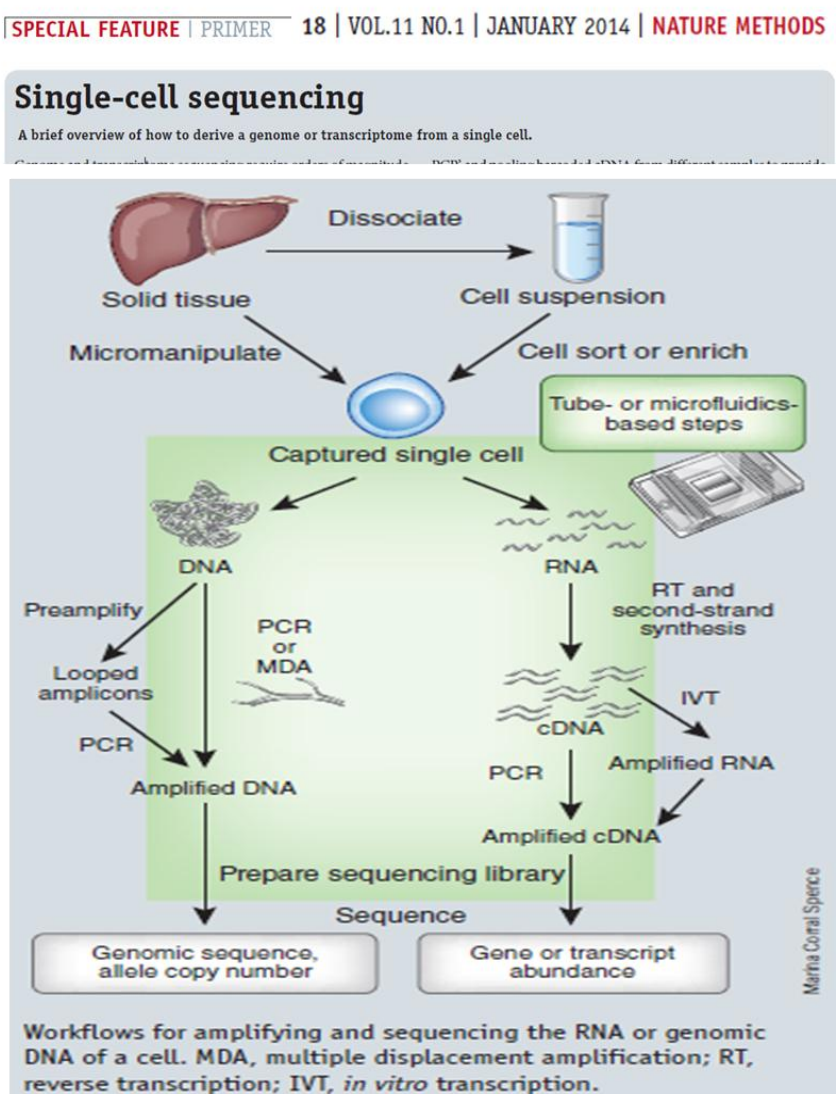


Figure 4 Illustrating correlation. Left: Red and yellow transcripts correlate on the single cell level. Right: Red and yellow transcripts correlate on sample level.

大多数细胞实验是在群体中分析基因活性，并没有关注细胞之间的个体差异。“这就如同拿房间里所有人的眼睛颜色进行平均，然后得出眼睛平均为灰色，但实际上可能房间里根本就没有人是灰眼睛。因此，理解和重视细胞群体中的个体差异是很有必要的。”

——Duke大学的副教授Benjamin Yellen

单细胞研究的技术路线



细胞样本的制备

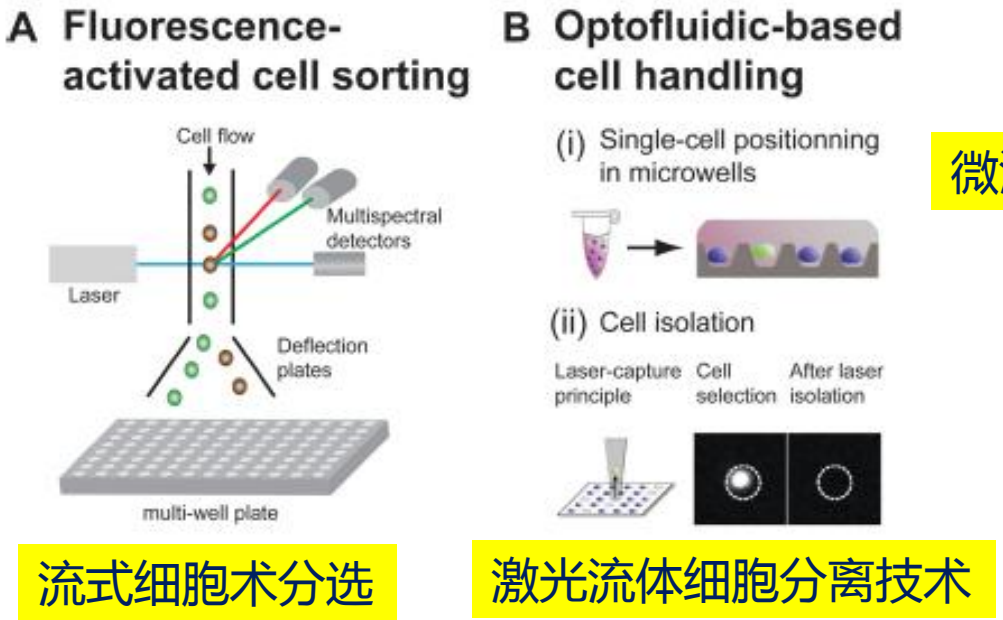
单细胞的捕获

单细胞全基因组和转录组测序

基因序列的研究

l people
y lives

单细胞获取方法



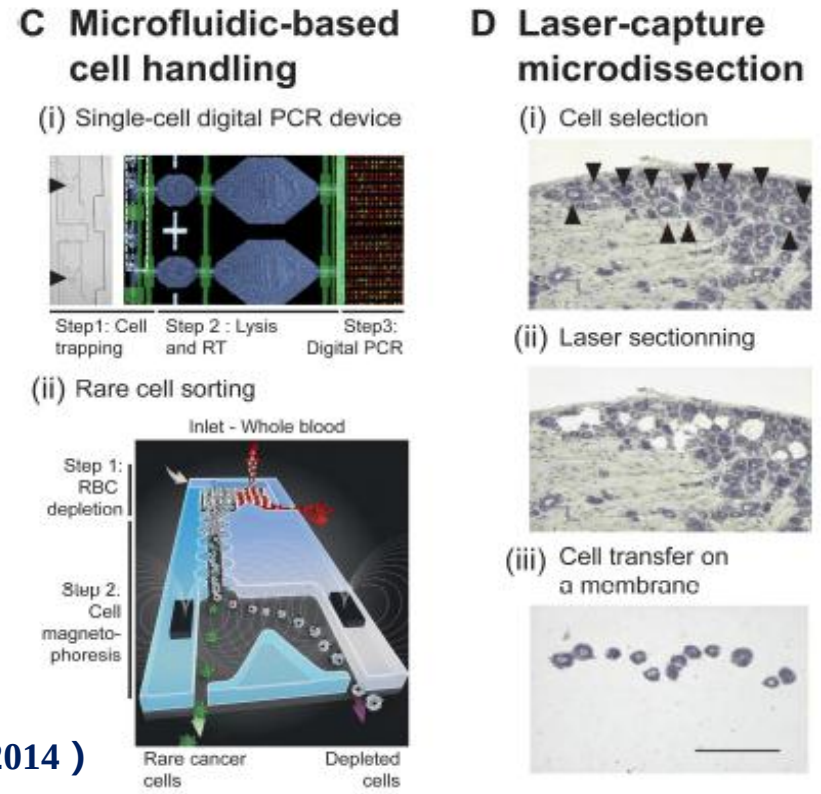
流式细胞术分选

激光流体细胞分离技术

最流行的策略

微液流的细胞分离技术

激光捕获显微切割



（Antoine-Emmanuel Saliba，etal，Nucleic Acids Research, 2014）

流式细胞仪作为单细胞获取的主流技术：

高通量

- 短时间内分析大量细胞。
- 高速分选，利于低比例细胞的快速富集。

多参数

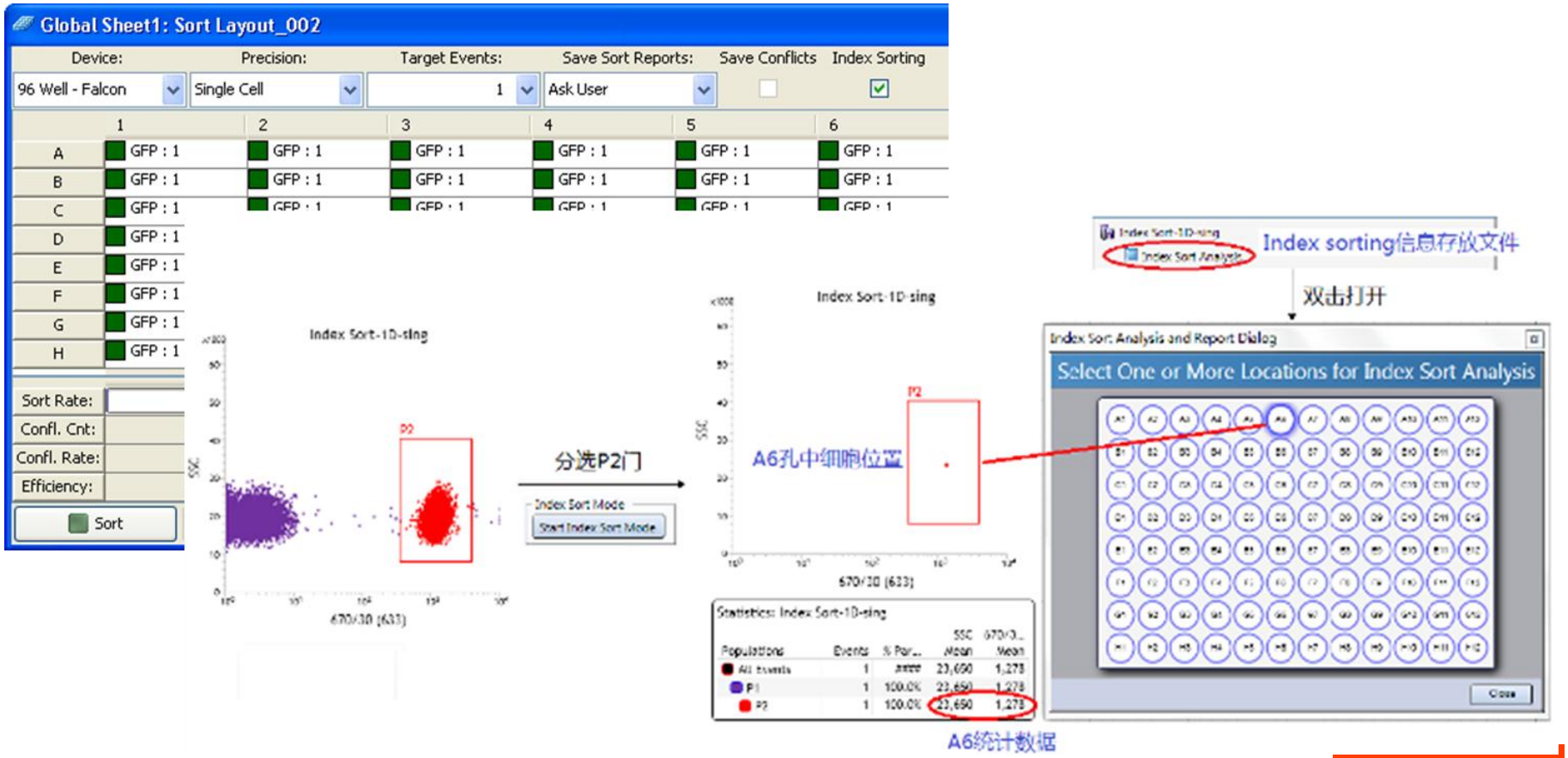
- 通过大小，颗粒度，内在或外在荧光染色，最大程度获得单细胞的多维信息。
- 将单个细胞的基因转录，蛋白表达和细胞功能三者有机结合，与上下游进行无缝衔接。

操作可行性

- 技术成熟，流程适合进行标准化操作。

Index sorting：索引分选功能

- 实时追踪单细胞分选结果，无需在显微镜下逐一筛选。
- 完美呈现细胞结构功能与基因表达调控的相互关系。



Helping all people live healthy lives

Development, Growth & Differentiation
The Japanese Society of Developmental Biologists
Develop. Growth Differ. (2010) 52, 131–144
doi: 10.1111/j.1440-169X.2009.01157.x

Original Article

Single-cell gene profiling of planarian stem cells using fluorescent activated cell sorting and its "index sorting" function for stem cell research

Tetsutaro Hayashi,[1,†] Norito Shibata,[1,2,†‡] Ryo Okumura,[3] Tomomi Kudome,[1] Osamu Nishimura,[1,‡] Hiroshi Tarui[1,*] and Kiyokazu Agata[1,*§]

独特应用：可与RT-PCR技术结合进行单个细胞水平的基因表达分析，将流式细胞分选技术与基因表型分析技术相结合， **一次实验即可获得单个细胞的基因水平、蛋白水平以及细胞水平的多重信息**，信息量更大、更全、更精确。

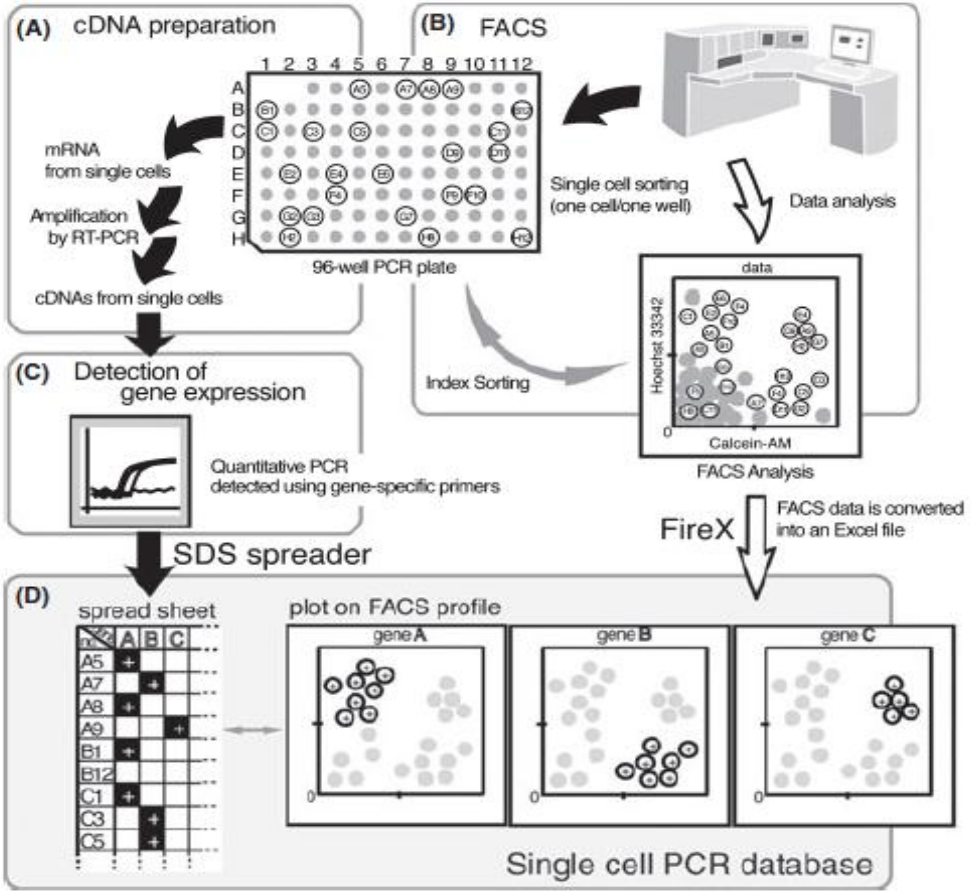


Fig. 1. Procedure for single-cell reverse transcription–polymerase chain reaction (RT–PCR) and index sorting analysis. For details of each panel, see text. FACS, fluorescent activated cell sorting; SDS, SDS file (SDS software: Applied Biosystems).

微生物基因测序面临的挑战：

微生物：泛指细菌、真菌、病毒等。

- 来源：自然环境中海洋、泥土、温泉及其它恶劣自然环境下，或人体外通腔道(如呼吸道、食道和肠道等)
- 特点：种类多，绝大多数或99%的微生物目前并不能在实验室培养生长

微生物基因测序：

- 混合材料测序：只能获得最优势生长的微生物的信息，无法获得一个完整的单一微生物基因组序列，不能提供足够的有用信息。
- 不能培养的微生物，达不到全基因组测序所需要的DNA质量。
- 这些阻碍了对微生物进行测序和研究。

单细胞测序技术的发展，无疑能够解决研究单个微生物细胞的基因组测序研究。

流式作为微生物细胞富集的重要技术手段

通过流式可以根据微生物的大小，颗粒度和DNA的含量，对不同的微生物进行分门别类和快速富集。

PROTOCOL

nature

Obtaining genomes from uncultivated environmental microorganisms using FACS–based single-cell genomics

Christian Rinke[1], Janey Lee[1], Nandita Nath[1], Danielle Goudeau[1], Brian Thompson[2], Nicole Poulton[2], Elizabeth Dmitrieff[2], Rex Malmstrom[1], Ramunas Stepanauskas[2] & Tanja Woyke[1]

[1]Department of Energy (DOE) Joint Genome Institute, Walnut Creek, California, USA. [2]Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA. Correspondence should be addressed to T.W. (twoyke@lbl.gov).

Published online 10 April 2014; doi:10.1038/nprot.2014.067

Single-cell genomics is a powerful tool for exploring the genetic makeup of environmental microorganisms, the vast majority of which are difficult, if not impossible, to cultivate with current approaches. Here we present a comprehensive protocol for obtaining genomes from uncultivated environmental microbes via high-throughput single-cell isolation by FACS. The protocol encompasses the preservation and pretreatment of differing environmental samples, followed by the physical separation, lysis, whole-genome amplification and 16S rRNA–based identification of individual bacterial and archaeal cells. The described procedure can be performed with standard molecular biology equipment and a FACS machine. It takes <12 h of bench time over a 4-d time period, and it generates up to 1 μg of genomic DNA from an individual microbial cell, which is suitable for downstream applications such as PCR amplification and shotgun sequencing. The completeness of the recovered genomes varies, with an average of ~50%.

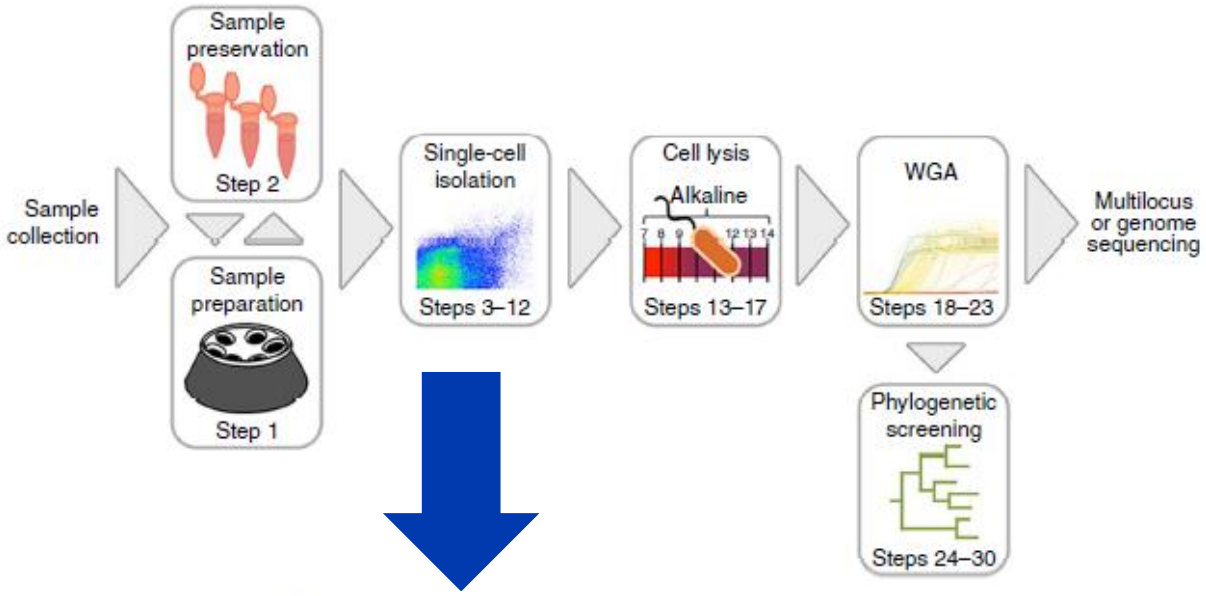


Figure 1 | Single-cell whole-genome amplification workflow. The workflow includes sample preparation (Step 1), sample preservation (betaine, glycerol; Step 2), single-cell isolation (flow cytometry; Steps 3–12), cell lysis (alkaline solution; Steps 13–17), WGA (MDA; Steps 18–23) and phylogenetic screening (16S rRNA or other marker genes; Steps 24–30). Specific amplified genomes then undergo multilocus or shotgun genome sequencing. Please note that only a small aliquot of DNA generated in the WGA is used for the phylogenetic screening and that phylogenetic reening is optional.

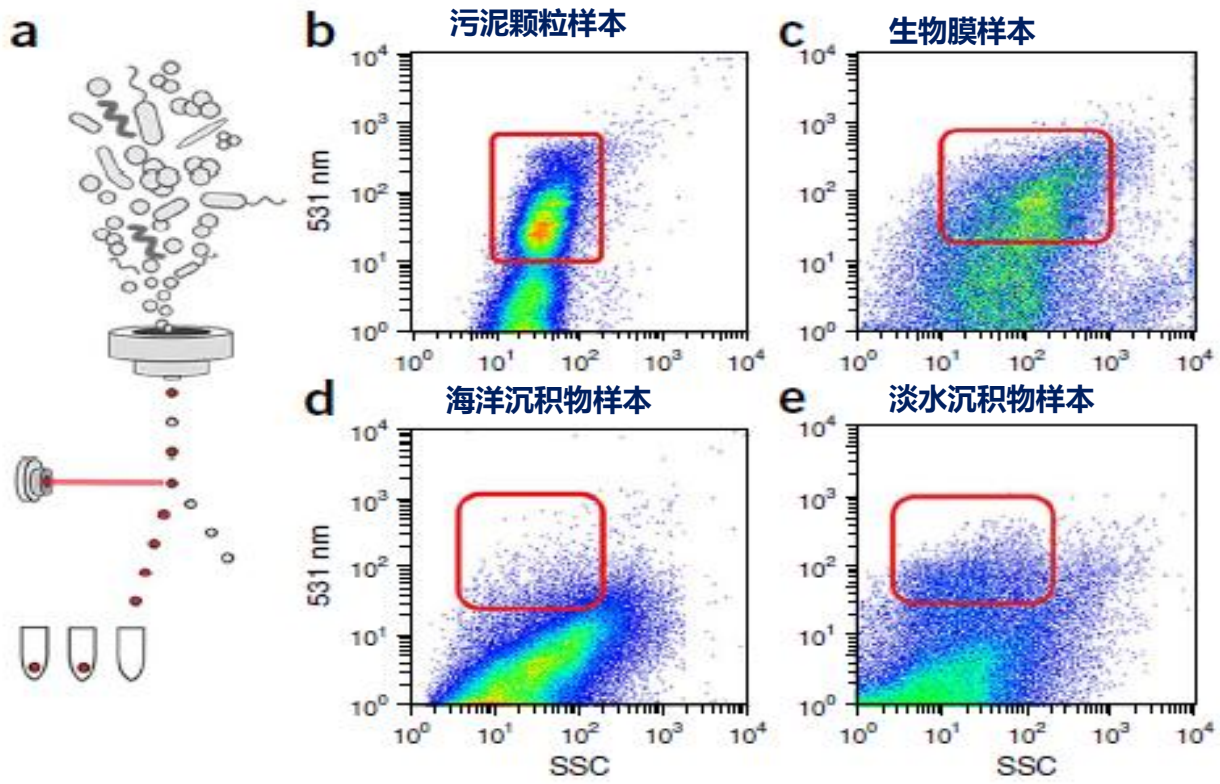
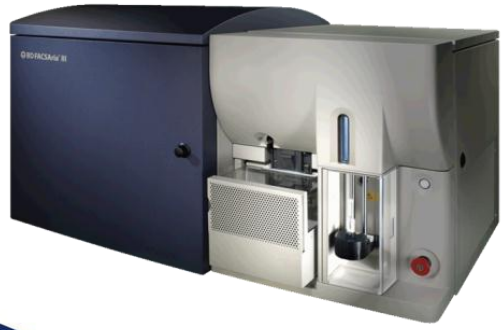


Figure 2 | High-throughput single-cell sorting. (**a**) Schematic drawing of the fluorescence-activated cell sorter. The environmental sample is arranged in a thin stream that moves one single cell at a time in front of the laser and detector, and the cells of interest are subsequently deflected into wells of a microtiter plate in a random manner, whereas the unwanted portion of the sample goes to waste. (**b**–**e**) Flow cytometry signals and sort windows (red outlines). (**b**) Granular sludge from a bioreactor, prepared as described in Step 1C. (**c**) Biofilm sample, prepared as described in Step 1B. (**d**,**e**) Marine sediment sample (**d**) and freshwater sediment sample (**e**), both prepared as described in Step 1A. The *x* axis shows the side scatter (SSC), and the *y* axis shows the relative green fluorescence of SYBR Green–stained samples at a 531-nm wavelength.

主要内容



水体微型生物

水生动物

环境毒理

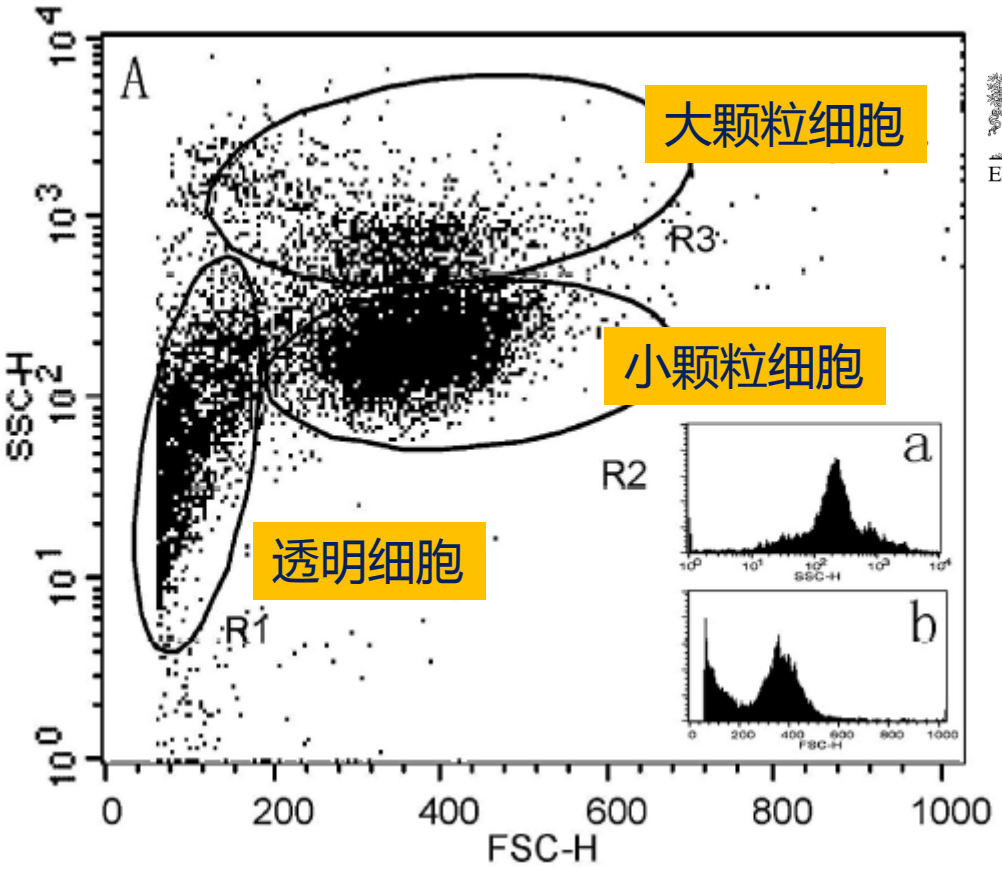
水生动物

- 水生动物细胞分类
- 水生动物细胞功能和机制研究
- 水生动物生物模型与遗传育种研究

1.细胞分类

- 在水生动物的研究中，主要集中在血细胞的分类研究。

（1）根据细胞大小和内部颗粒复杂程度

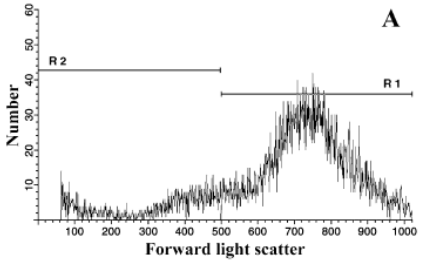


墨吉对虾血细胞的流式细胞术分群

ELSEVIER
Fish & Shellfish Immunology 17 (2004) 223–233
Fish & Shellfish Immunology
www.elsevier.com/locate/fsi

Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides

Washington Cárdenas[a,1], John R. Dankert[a], Jill A. Jenkins[b,*]
[a]Department of Biology, University of Louisiana, Lafayette, LA 70504, USA
[b]U.S. Geological Survey, National Wetlands Research Center, 700 Cajundome Boulevard, Lafayette, LA 70506, USA



小龙虾血细胞在体外添加酵母聚糖和脂多糖后细胞大小的变化。

细胞分类

（2）带有特异性抗原的细胞

——单克隆抗体与荧光染料相结合

Production, characterization and application of monoclonal antibody to spherulocytes: A subpopulation of coelomocytes of *Apostichopus japonicus*

Qiang Li [a], Ying Li [a], Hua Li [a,*], Yi-Nan Wang [a], De-Hai Xu [b]

[a] Key Laboratory of Mariculture, Agriculture Ministry, PRC, Dalian Ocean University, Dalian 116023, China
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仿刺参体腔细胞

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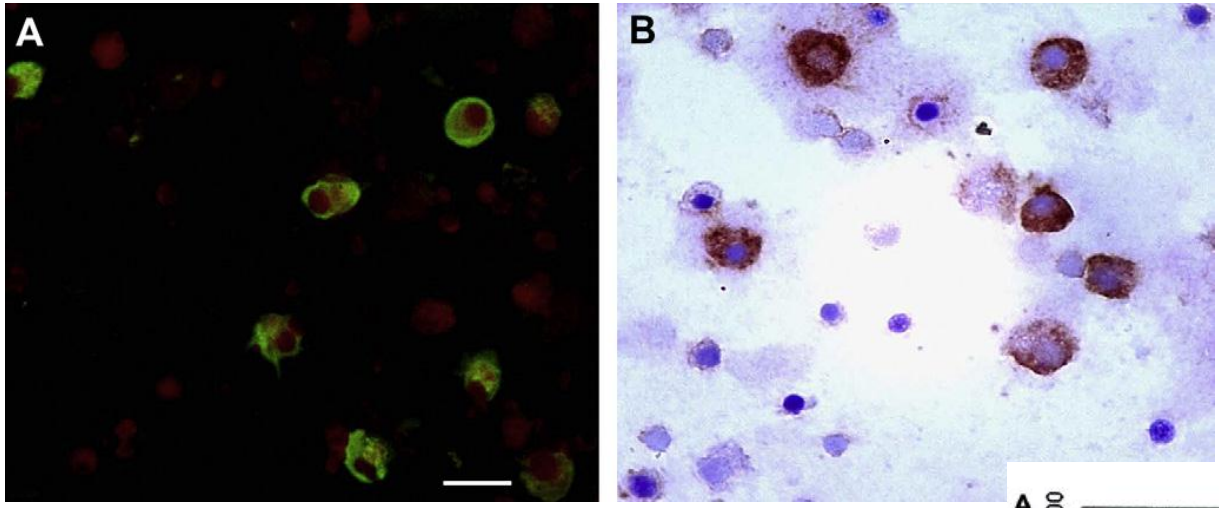
Monoclonal antibody

ABSTRACT

One monoclonal antibody (mAb 3F6) against coelomocytes of sea cucumber *Apostichopus japonicus* was developed by immunization of Balb/C mice. Analyzed by indirect immunofluorescence assay test (IIFAT), immunocytochemical assay (ICA), Western blotting and fluorescenceactivated cell sorter (FACS), mAb 3F6 showed specific for spherulocytes of *A. japonicus*. The mAb 3F6 recognized an antigen of molecular weight 136 kDa in Western blotting. Isotype analysis revealed mAb 3F6 as IgG type. The flow cytometry assay confirmed the microscopy observations and showed coelomocytes positive to mAb 3F6. The antigencity of haemocytes or coelomocytes of *Hemicentrotus pulcherrimus*, *Scapharca subcrenata*, *Asterina pectinifera*, *Asterias rollestoni*, *Ruditapes philipinarum*, *Patinopecten yessoensis* and *Mytilus edulis* was compared and the result showed that none of them was positive with mAb 3F6. Most of cells free in polian vesicle were positive with mAb 3F6. The positive cells are in spherical shape, 5–7 μm in diameter, smaller than coelomic spherulocytes of *A. japonicus*. The result of immunofluorescent staining with cells in hemal vessel showed that there were strong positive signals on cytoplasm of some spherical cells with diameter of 7–8 μm. Some other cells with higher nucelo-plasmic ratio, about 5–6 μm in diameter showed weak positive signals on membrane. Immunohistochemistry assay revealed that positive signals were mostly observed in the lumen structure of rete mirabile and haemocoel of the respiratory tree. In addition, the outer epidermis of body wall and tentacle also showed positive.

细胞分类

Detection of coelomocytes reacted with mAb 3F6 by indirect immunofluorescence assay test



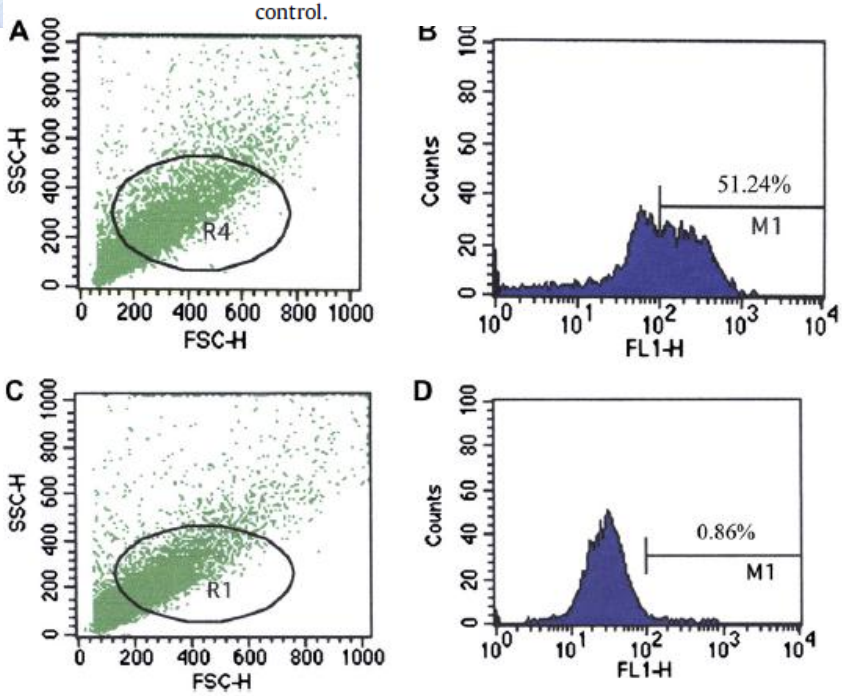
2.7. FACS analysis

Coelomic fluid was drawn from healthy *A. japonicus* and was immediately diluted (1:3) in MAS. The coelomocytes were washed twice with PBS, centrifuged at 800 × *g*, and then resuspended in PBS. The anti-coelomocytes mAb was added to coelomocytes at the dilution of 1:10 and incubated for 1 h at 37 °C. Subsequently, the cells were washed three times with PBS and incubated with GAM-FITC diluted 1:256 in PBS for 1 h at 37 °C. After washes, the coelomocytes were analyzed with an FACscan (Becton Dickinson, FACSCalibur™). Myeloma culture supernatant was used as negative control.

Detection reaction of mAb 3F6 with coelomocytes by FACS.

3.3. FACS analysis

At least 20,000 cells were counted in the flow cytometry assay. The results were presented as cell cytograms using a dot plot combination of low angle forward scattered (FSC) and right angle scattered (SSC) laser light and FITC fluorescence histograms of immunostained cells (Fig. 3). A 51.24% of coelomocytes labeled with mAb 3F6 was counted by the cytometer. The result of flow cytometry assay in this study confirmed the microscopy findings and demonstrated coelomocytes positive to mAb 3F6.



2. 细胞功能和机制研究

细胞活性分析：根据膜的完整性；

- 常用染料：PI

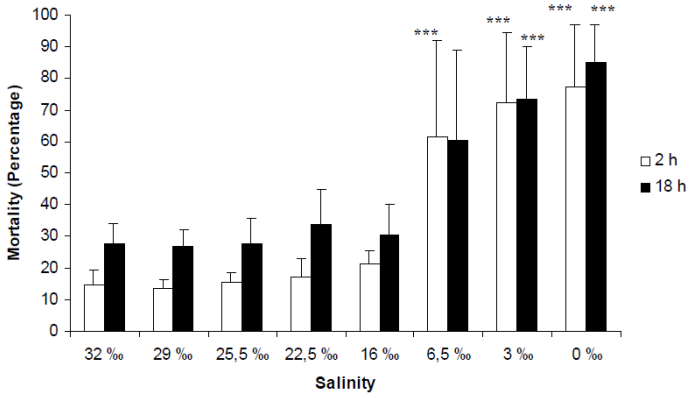
Effects of temperature and salinity on haemocyte activities of the Pacific oyster, Crassostrea gigas (Thunberg)

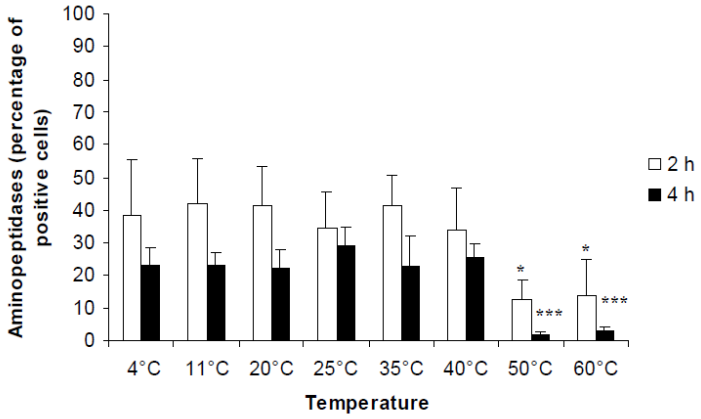
Beatrice Gagnaire[a], Heloise Frouin[a, b], Kevin Moreau[a], Helene Thomas-Guyon[b] and Tristan Renault[a*]

[a]Laboratoire de Génétique et Pathologie (LGP), Ronce-les-Bains, IFREMER La Tremblade, 17390 La Tremblade, France
[b]Laboratoire de Biologie et Environnement Marin (LBEM), Université de La Rochelle, FRE 2727, Avenue Michel Crépeau, 17042 La Rochelle, France

*: Corresponding author : Tel.: +33 5 4676 2649; fax: +33 5 4676 2611. trenault@ifremer.fr

太平洋牡蛎血细胞





利用PI检测不同温度和盐度对太平洋牡蛎血细胞分别在活体内和体外环境下活性的影响。

In vitro effects of cadmium and mercury on Pacific oyster, Crassostrea gigas (Thunberg), haemocytes

B. Gagnaire[a], H. Thomas-Guyon[b] and T. Renault[a]*

[a] IFREMER, Laboratoire de Génétique et Pathologie (LGP), 17390, La Tremblade, France
[b] Université de La Rochelle, Laboratoire de Biologie et Environnement Marin (LBEM), avenue Michel Crépeau, 17042, La Rochelle, France

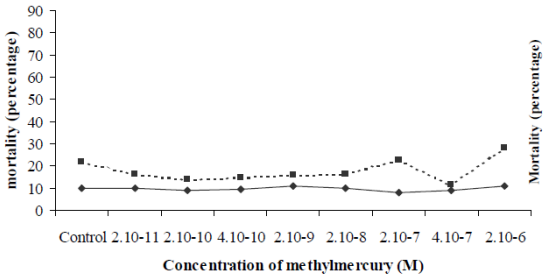
* trenault@ifremer.fr IFREMER, Laboratoire de Génétique et Pathologie (LPG), 17390 La Tremblade, France. Tel.: +33-5-46-36-98-41; fax: +33-5-46-36-37-51.

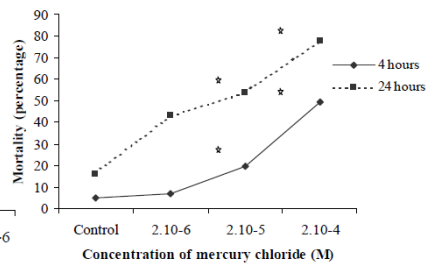
Tableau 1 : Haemocyte mortality tested by flow cytometry with increasing concentrations of cadmium *in vitro* (0-3.10^{-11}-3.10^{-10}-3.10^{-9}-3.10^{-8}-3.10^{-7}-3.10^{-6}-3.10^{-5}-3.10^{-4} M) after 4 and 24 h incubation at 20°C. Values are averages of two replicates.

	Control	3.10-11 M	3.10-10 M	3.10-9 M	3.10-8 M	3.10-7 M	3.10-6 M	3.10-5 M	3.10-4 M
4h	9,3	9,4	9,0	10,7	9,7	9,2	9,6	10,5	10,7
24h	12,5	13,5	20,1	19,7	31,8	23,9	18,4	30,8	17,0

使用PI进行流式检测，评估不同浓度的镉和汞对离体太平洋牡蛎血细胞活性的影响。

nocyte mortality tested by flow cytometry with increasing concentrations of mercury *in vitro* after 4 and 24 h incubation at 20°C. Values are averages of three replicates





2.细胞功能和机制研究

细胞周期和倍性分析

- **原理**：对细胞周期的分析主要通过检测细胞的总DNA含量。

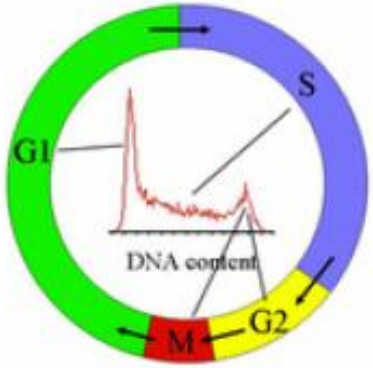
  倍性是指生物单倍体基因组所含DNA的总量。

染色计数

传统方法
费时费力
不能活体检测

流式细胞仪

批量处理
节约时间
结果稳定



用核酸染料对DNA进行染色，根据荧光的强弱可区分出G_0/G_1期(二倍体)、S期(超二倍体)、G_2/M期(四倍体)，分析各期细胞数量的比例即可知细胞的增殖状况。

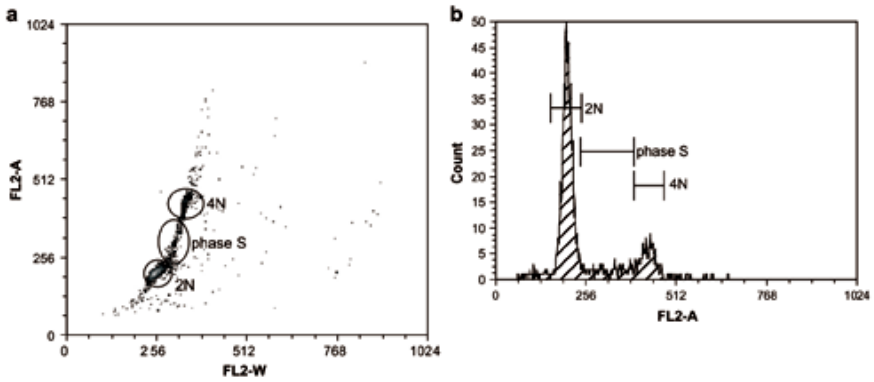
- **应用价值**：

A.细胞生物学基础研究

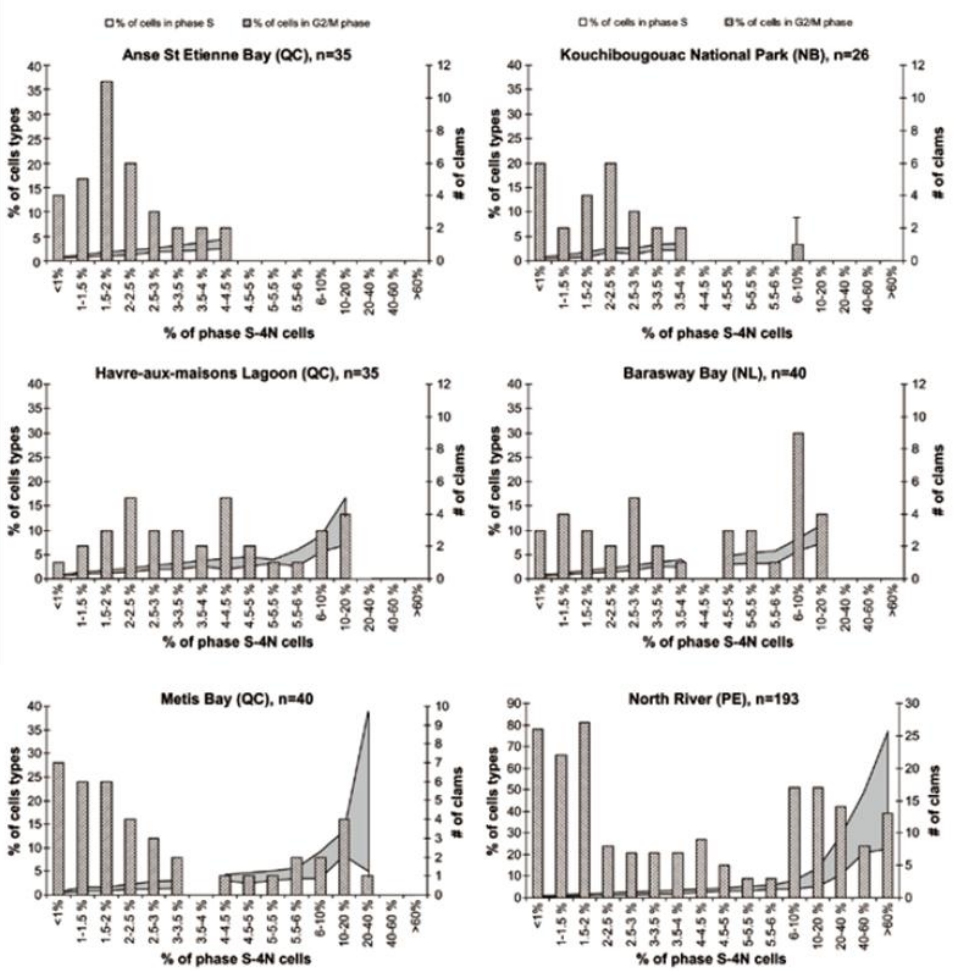
B.遗传育种

C.种群多样性

应用举例：细胞周期和倍性分析



利用流式细胞仪FACSCalibur分析砂海螂血细胞中正常二倍体(G0/G1期)、S期和四倍体细胞(G2/M期)。



不同采集点砂海螂种群血细胞的细胞周期分析结果。

Assessment of haemic neoplasia in different soft shell clam Mya arenaria populations from eastern Canada by flow cytometry

Maryse Delaporte, Stéphanie Synard, et al. Journal of Invertebrate Pathology (2006) Vol. 93: 190-197

应用举例：基因组大小

Flow Cytometry Measurement of the DNA Contents of G0/G1 Diploid Cells From Three Different Teleost Fish Species

Juana Ciudad,[1] Elena Cid,[2] Almudena Velasco,[2] Juan M. Lara,[2] José Aijón,[2] and Alberto Orfao[1]
[1]Servicio de Citometría, Universidad de Salamanca, Salamanca, Spain
[2]Biología Celular, INCyL, Universidad de Salamanca, Salamanca, Spain

Received 9 July 2001; Revision Received 7 February 2002; Accepted 15 February 2002

Background: Although there is a lot information in the literature about genome size in fish, a high variability among data for the same species is reported, being mainly related to methodological aspects. Flow cytometry-based fluorescence measurements of intercalating dyes is the most attractiv
high speed, a
Methods: We
nuclei of th
Tinca tinca,
Forty-three a
cells were an
ciated fluores
flow cytomet
as a referenc

Results: Our results show that *C. auratus* (3.584 ± 0.058 pg per nucleus) and *D. rerio* (3.357 ± 0.074 pg per nucleus) showed similar DNA contents per cell, whereas it was significantly lower (2.398 ± 0.038 pg per nucleus) in *T. tinca*. Interestingly, a low intraspecies variability was

Flow cytometric determination of genome size in European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), thinlip mullet (*Liza ramada*), and European eel (*Anguilla anguilla*)

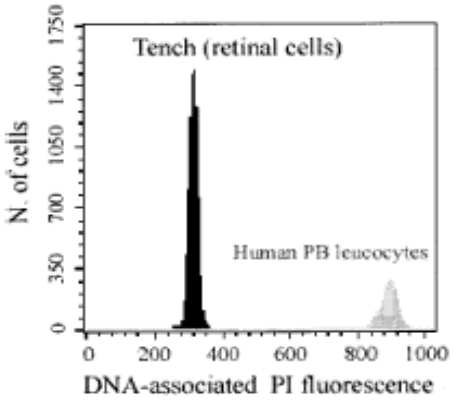
Stefano Peruzzi[1,2,a], Béatrice Chatain[1] and Bruno Menu[1]

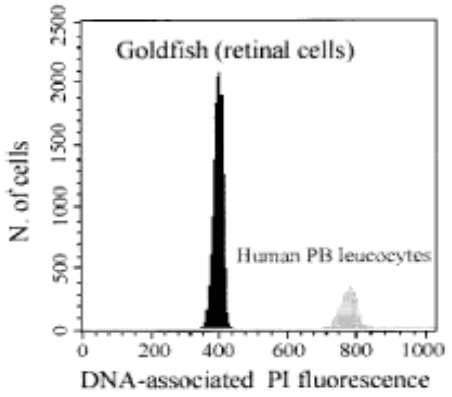
[1] IFREMER, Laboratoire de Recherche Piscicole de Méditerranée, Chemin de Maguelone, 34250 Palavas-les-Flots, France
[2] Norwegian College of Fishery Science, University of Tromsø, 9037 Tromsø, Norway

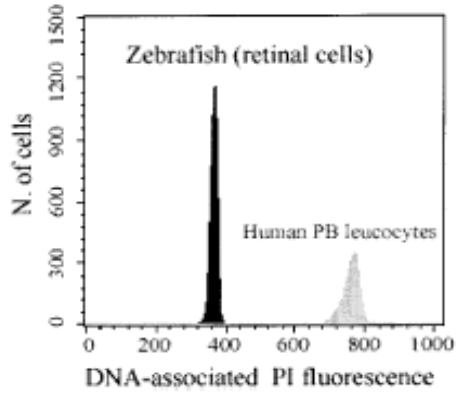
Received 19 December 2003; Accepted 5 July 2004

Abstract – The nuclear DNA content in *Dicentrarchus labrax*, *Sparus aurata*, *Liza ramada*, and *Anguilla anguilla*, was measured by flow cytometric analysis. Male human leukocytes were used as internal reference cells. Nucleated fish erythrocytes were stained simultaneously to human leukocytes with a staining buffer containing propidium iodide (PI). Nuclear DNA contents of target species were estimated in relation to an assigned value of 7.0 pg DNA for male human leukocytes. The DNA content/nucleus (±CI) was 1.55 ± 0.02 pg for *D. labrax*, 1.90 ± 0.03 pg for *S. aurata*, 1.57 ± 0.02 pg for *L. ramada*, and 2.43 ± 0.04 pg for *A. anguilla*. Intraspecific DNA content variations ranged from 0–9% and averaged around 4% in all taxa. The results did not evidence significant differences in genome size between males and females in either *D. labrax* or *L. ramada*.

Key words: DNA content / Teleosts / *Dicentrarchus labrax* / *Sparus aurata* / *Liza ramada* / *Anguilla anguilla*





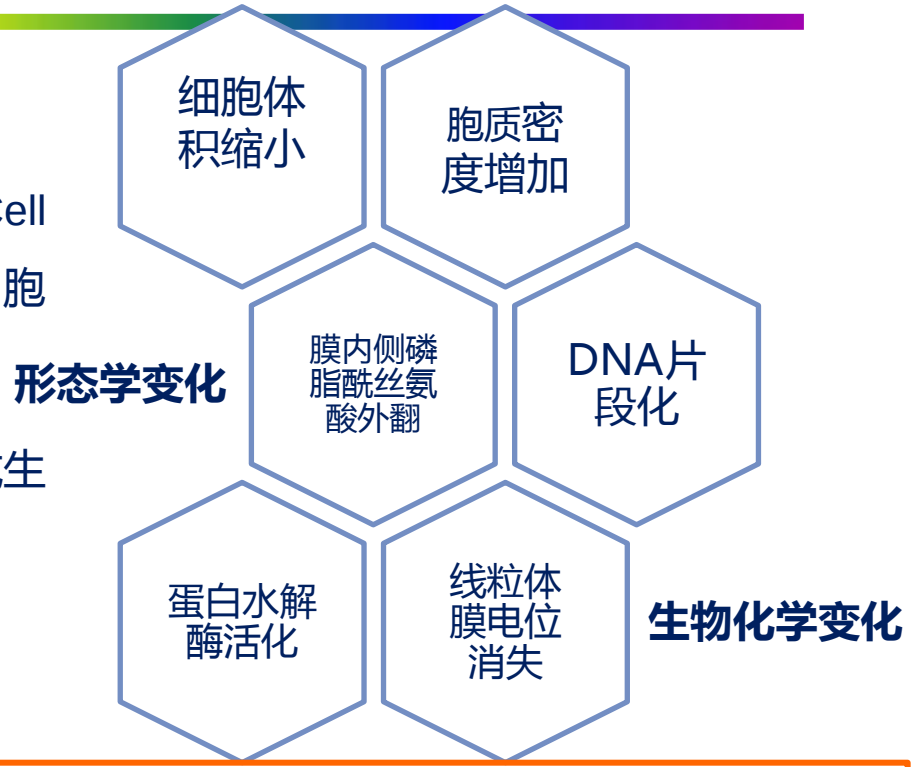


2. 细胞功能和机制研究

细胞凋亡检测

定义：又称为程序性细胞死亡(Programmed Cell Death)是机体主动的、高度有序地清除无用细胞的过程。

细胞坏死（ Necrosis ）是细胞受到强烈理化或生物因素作用引起细胞无序变化的死亡过程。

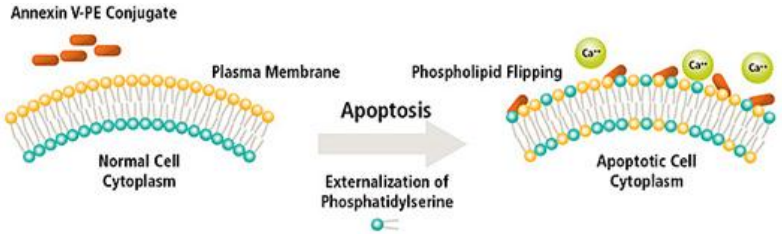


传统检测凋亡的方法：

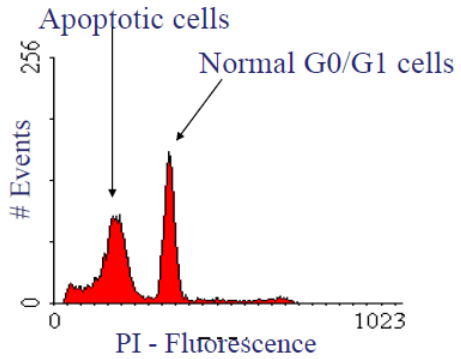
- **显微镜下进行形态学的观察；**
- **细胞DNA提取物的电泳实验；**
- **细胞内凋亡信号通路相关蛋白的表达研究（如ELISA, IHC, Western blot）；**

细胞凋亡的流式方法学

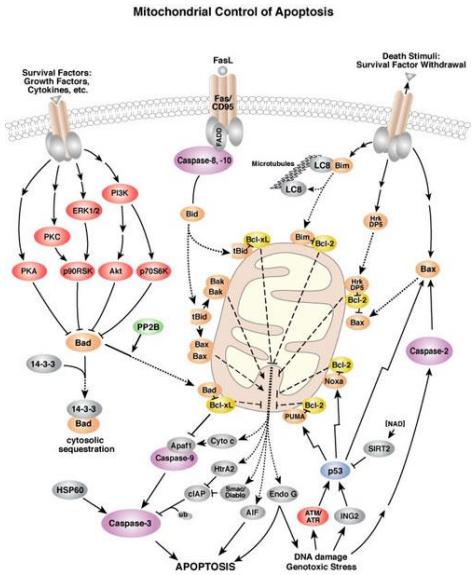
Annexin V/PI 双染色法



凋亡峰检测(PI)



线粒体膜电位

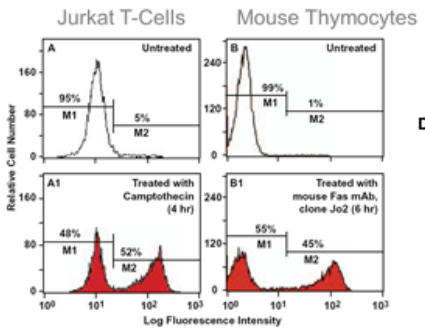


相关凋亡酶的检测

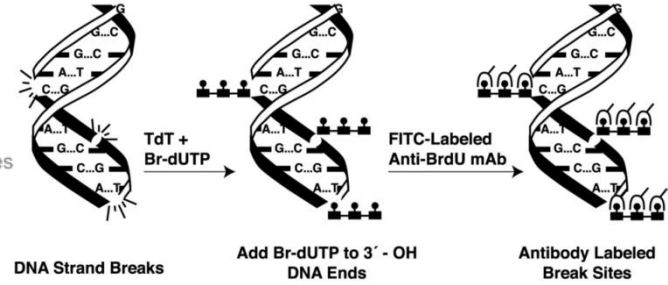
- **原理：**特异性抗原抗体反应
- **荧光染料：**荧光素-anti caspase-3
- **检测方法：**胞内染色

检测试剂盒：

Kit	Cat. No
Caspase-3, Active Form, mAb Apoptosis Kit : FITC	550480
Caspase-3, Active Form, pAb Apoptosis Kit (PE)	550914



TUNEL检测（末端转移酶标记）



应用举例：细胞凋亡

Quantitative assessment of apoptotic hemocytes in white spot syndrome virus (WSSV)-infected penaeid shrimp, *Penaeus monodon* and *Penaeus indicus*, by flow cytometric analysis

A.S. Sahul Hameed *, M. Sarathi, R. Sudhakaran, G. Balasubramanian, S. Syed Musthaq

Aquaculture Biotechnology Division, Department of Zoology, C.Abdul Hakeem College, Melvisharam-632 509 Vellore Dt., T.N., India

Received 3 September 2005; received in revised form 1 February 2006; accepted 21 February 2006

Abstract

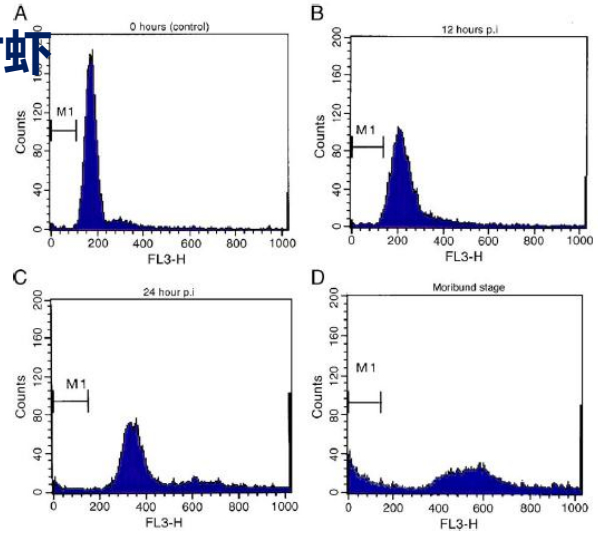
A study was carried out to determine the percentage of apoptotic hemocytes in WSSV-infected *Penaeus monodon* and *Penaeus indicus* at different time intervals by DNA fragmentation assay using flow cytometry and agarose gel electrophoresis. Shrimp were injected with WSSV, and hemolymph at different time intervals was collected to determine apoptotic hemocytes. Apoptotic hemocytes were increased in course of infection and were found to be higher in *P. indicus* in comparison with *P. monodon*. Furthermore, DNA fragmentation of WSSV-injected hemocytes was also evaluated by the agarose gel electrophoresis and characteristic intranucleosomal DNA ladder patterns were detected in the DNA extracted from hemocytes of WSSV-infected shrimp, but not in the DNA of hemocytes from uninfected shrimp.

Keywords: White spot syndrome virus; Shrimp; Apoptosis; Hemocytes; DNA fragmentation assay; Flow cytometry

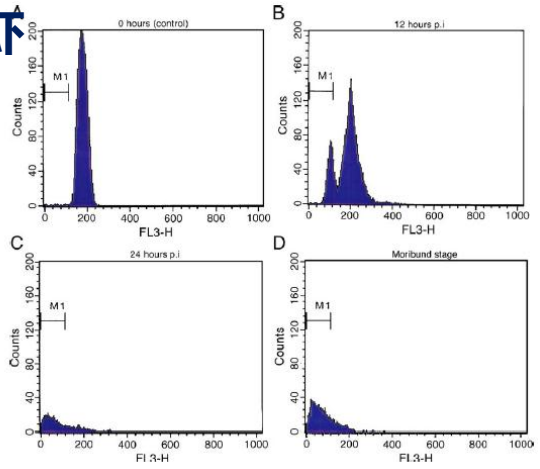
使用流式检测斑节对虾(*P．monodon*)和印度对虾(*P．indicus*)经白斑综合症病毒注射后的血细胞凋亡情况。

感染病毒之后细胞凋亡情况

斑节对虾



印度对虾



流式结果与DNA电泳结果相匹配

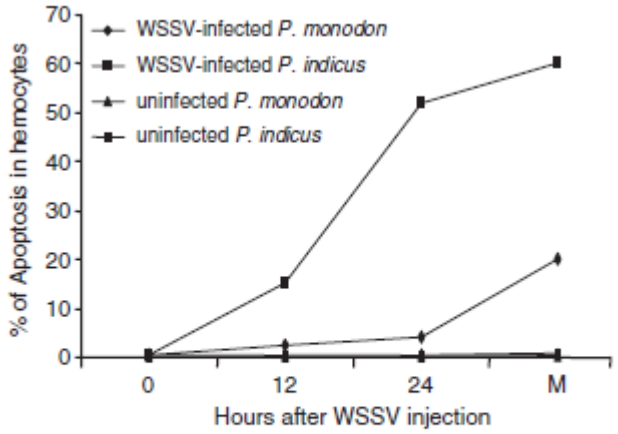


Fig. 6. Percentage of apoptosis in healthy and WSSV-infected *P. monodon* and *P. indicus* (M – moribund).

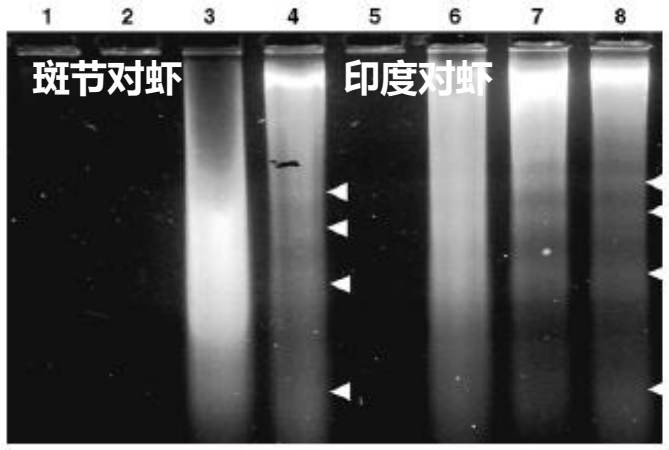


Fig. 7. DNA laddering from WSSV-infected penaeid shrimp, *P. monodon* (lanes 1 to 4) and *P. indicus* (lanes 5 to 8) at different time intervals. DNA fragments are shown by an arrowhead. Lanes 1 and 5 – 0 h p.i.; lanes 2 and 6 – 12h p.i.; lanes 3 and 7 – 24h p.i.; lanes 4 and 8 – moribund.

2. 细胞功能和机制研究

细胞钙离子检测

细胞内钙离子浓度对细胞许多功能如酶的活性起着主要作用，还是重要的细胞内信使，测定细胞内钙离子的变化，对于了解细胞内信息传递，细胞凋亡等都有一定的意义。

文章比较研究了太平洋牡蛎离体血细胞在血淋巴和人工海水中孵育后钙离子含量的差异。

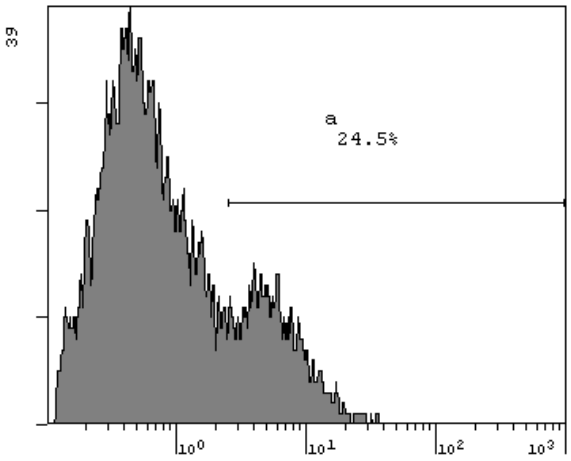


Fig. 6. Cytogram showing Fluo-3/AM labelled haemocytes monitored by Percentage "a" correspond to cells stained by the probe (positive). X-a fluorescence (log scale); Y-axis: number of events.

A flow cytometric approach to study intracellular-free Ca2+ in Crassostrea gigas haemocytes

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[b]IFREMER La Tremblade, Laboratoire de Génétique et Pathologie (LGP), Ronce-les-Bains, 17390 La Tremblade, France
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Abstract: Bivalve haemocytes are essential in defence mechanisms including phagocytosis. They also produce molecules including hydrolytic enzymes and antimicrobial peptides that contribute to pathogen destruction. Although haemocyte activities have been extensively studied, relatively little is known about the intracellular signalling pathways that are evoked during haemocyte activation and especially the role of calcium.

Flow cytometry has been used for the first time to define the effect of cell incubation in haemolymph and artificial sea water (ASW) on Pacific oyster, Crassostrea gigas, haemocytes. Cell viability, enzymatic activities (esterases and aminopeptidases), phagocytosis and granulocyte percentage were analysed. Viability and some activities were different in haemolymph and ASW. Cytoplasmic-free calcium in circulating haemocytes was then investigated by flow cytometry in both media using a calcium probe (Fluo-3/AM). To explore calcium homeostasis, different calcium modulators were tested. The calcium chelator Bapta/AM (10 µM) reduced significantly the percentage of Fluo-3-positive cells in ASW. In addition, ryanodine (5 µM) induced a significant enhancement of the percentage of Fluo-3 positive cells in haemolymph and in ASW. Flow cytometry may be used to study calcium movements in C. gigas haemocytes, but several haemocyte incubation media need to be tested in order to confirm results. The objective of the study should be considered before selecting a particular experimental medium.

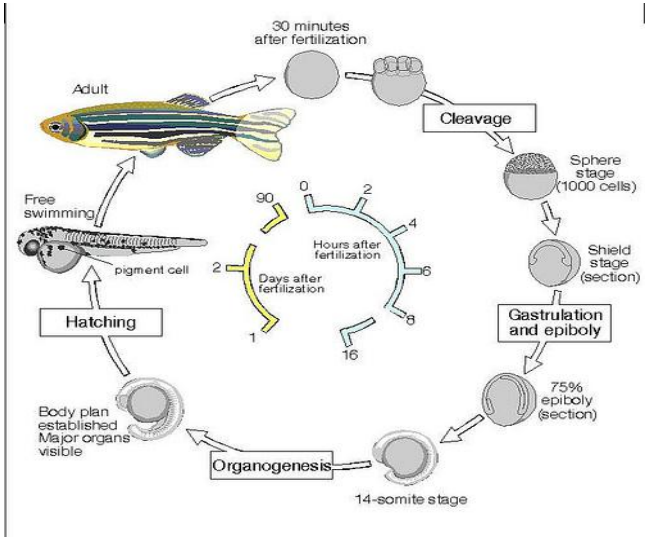
水生动物

- 水生动物细胞分类
- 水生动物细胞功能和机制研究
- 水生动物生物模型与遗传育种研究
  1. 模式生物：斑马鱼
  2. 水生生物精子质量评估

1.模式生物：斑马鱼

CZRC 国家斑马鱼资源中心
CHINA ZEBRAFISH RESOURCE CENTER

转基因斑马鱼技术服务平台



斑马鱼作为重要的脊椎动物模式系统之一，由于其体型小，世代周期短，繁殖率高，饲养管理康价方便且成本低。其胚胎透明，且在体外受精和发育等多方面的优势，使其在毒理学研究领域发挥着越来越重要的作用。目前它已被广泛地用于胚胎发育毒理学、病理毒理学、环境毒理学等毒理学领域的研究中，并展现出其特有的优势。

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模式生物：斑马鱼

- 斑马鱼的研究现状

贾顺姬等：中国斑马鱼研究发展历程及现状

1. 我国斑马鱼研究的SCI源刊论文发表数在世界范围内的比重及相关研究机构的数量迅速增加。

2.国内以斑马鱼为模式动物的研究内容更加丰富，领域更加广阔，并与现代生命科学研究的热点紧密契合。

3. 我国斑马鱼相关研究的水平明显提升，一些研究成果具有非常重要的科学价值，甚至达到世界先进水平，得到世界同行的认可。

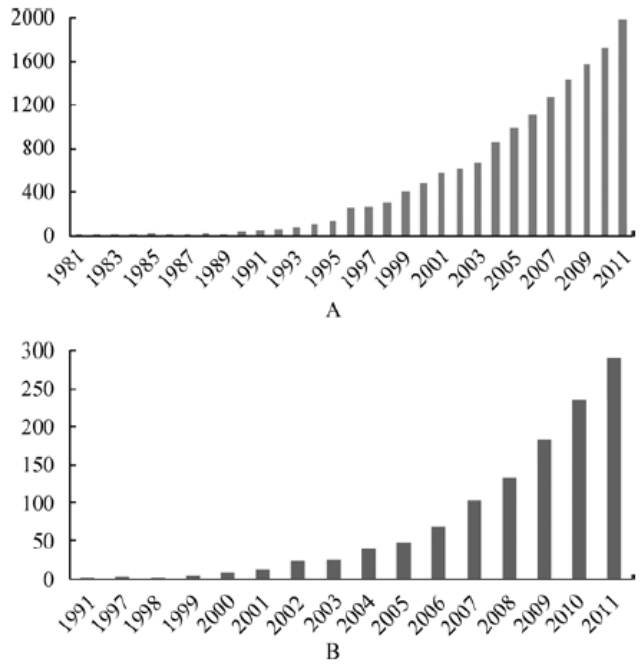


图 1　斑马鱼相关 SCI 论文年度统计图

A：1981~2011 年，世界范围内发表的 SCI 论文统计结果；B：1991~2011 年，中国的研究机构参与发表的 SCI 论文统计结果。以 Web of Science[SM] 数据库为检索依据。

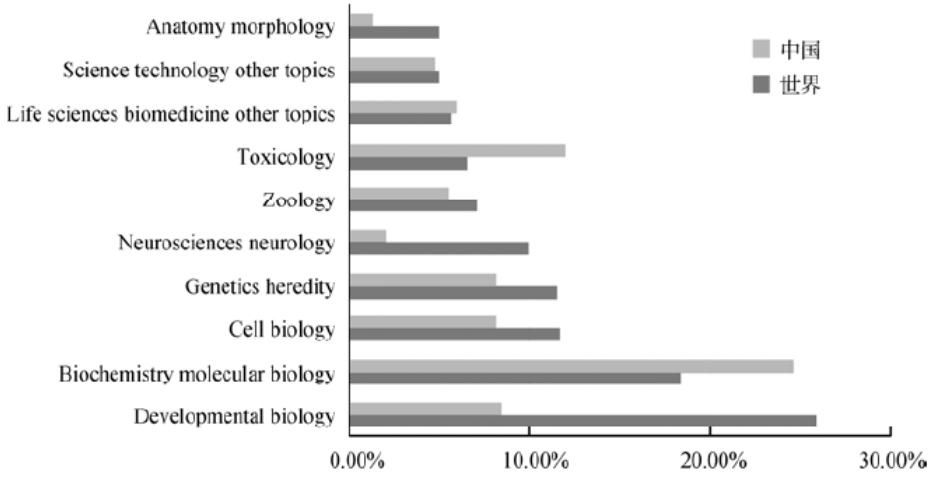


图 3　斑马鱼相关 SCI 论文所涉及的主要学科领域，中国与世界整体水平的比较结果

应用举例

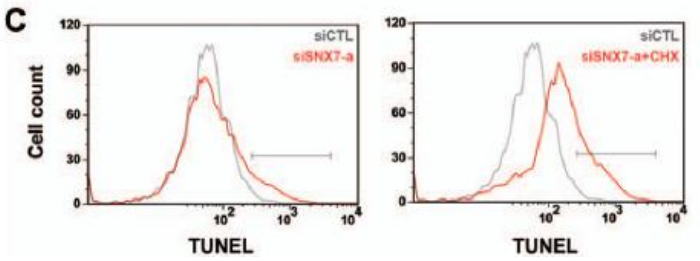
- 斑马鱼作为研究生长发育及相关信号通路的生物模型
- 斑马鱼作为疾病机制研究的生物模型
- 斑马鱼作为毒理模式动物研究外源毒物作用机制

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

An Antiapoptotic Role of Sorting Nexin 7 Is Requir for Liver Development in Zebrafish

Liangliang Xu,[1,2] Wenguang Yin,[2] Jianhong Xia,[2] Meixiu Peng,[2] Song Li,[3] Shuo Lin,[3] Duanqing Pei,[2] and Xiaodong Shu[2]

Sorting nexin (SNX) family proteins are best characterized for their abilities to regulate protein trafficking during processes such as endocytosis of membrane receptors, endosomal sorting, and protein degradation, but their *in vivo* functions remain largely unknown. We started to investigate the biological functions of SNXs using the zebrafish model. In this study, we demonstrated that SNX7 was essential for embryonic liver development. Hepatoblasts were specified normally, and the proliferation of these cells was not affected when SNX7 was knocked down by gene-specific morpholinos; however, they underwent massive apoptosis during the early budding stage. SNX7 mainly regulated the survival of cells in the embryonic liver and did not affect the viability of cells in other endoderm-derived organs. We further demonstrated that down-regulation of SNX7 by short interfering RNAs induced apoptosis in cell culture. At the molecular level, the cellular FLICE-like inhibitory protein (c-FLIP)/caspase 8 pathway was activated when SNX7 was down-regulated. Furthermore, overexpression of c-FLIP$_S$ was able to rescue the SNX7 knock-down-induced liver defect. *Conclusion*: SNX7 is a liver-enriched antiapoptotic protein that is indispensable for the survival of hepatoblasts during zebrafish early embryogenesis.
(Hepatology 2012;55:1985-1993)



广州生物医药与健康研究院裴端卿教授和舒晓东教授合作利用斑马鱼作为肝脏发育的研究模型，揭示了SNX7通过抑制细胞凋亡调控肝脏形成的作用机制。

Nucleic Acids Research Advance Access published August 25, 2011

Nucleic Acids Research, 2011, 1–12
doi:10.1093/nar/gkr645

Transgenic zebrafish model to study translational control mediated by upstream open reading frame of human *chop* gene

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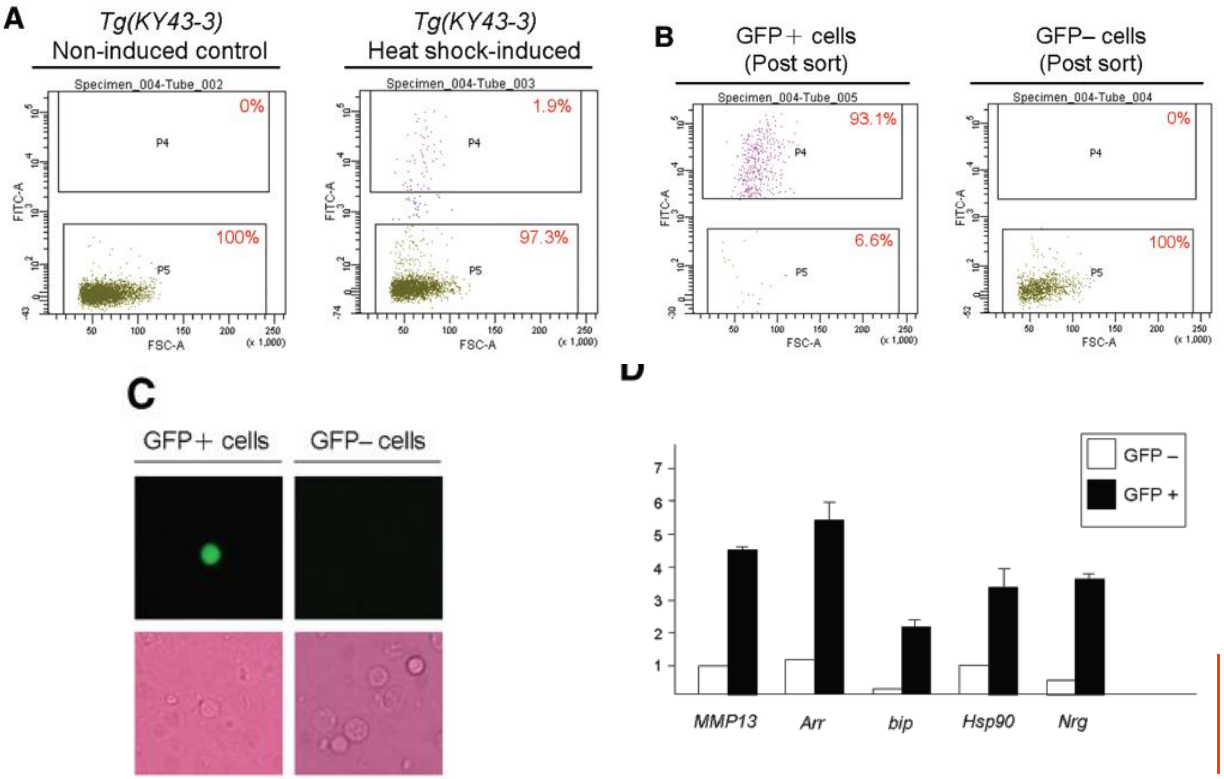
[1]Institute of Molecular and Cellular Biology, College of Life Science, National Taiwan University, [2]Institute of Molecular Medicine, College of Medicine, National Taiwan University and [3]Taipei Municipal Jianguo High School, Taipei, Taiwan

Received January 13, 2011; Revised July 21, 2011; Accepte

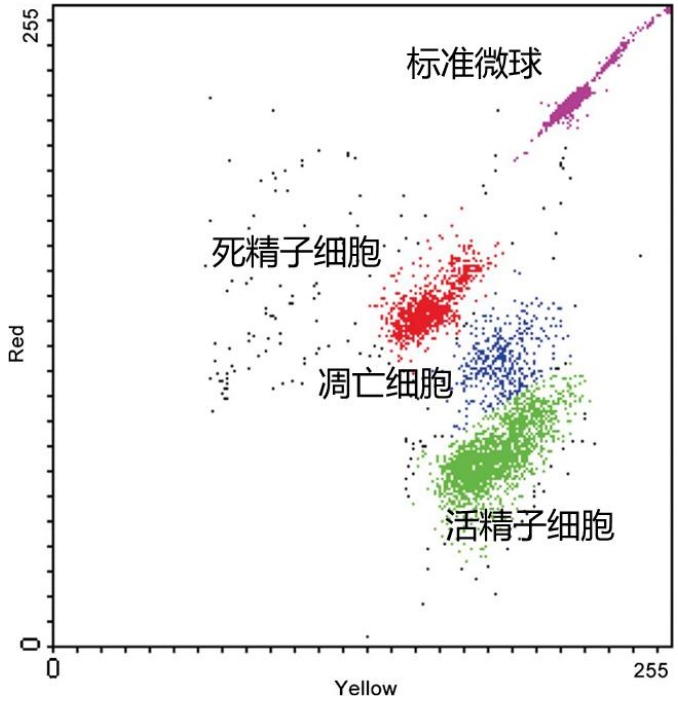
Dissociation of embryonic cells and FACS

Four hundred embryos at 120 hpf from the huORFZ lin were collected into a 2-ml Eppendorf, resuspended witl 1.2 ml pre-heated (28°C) protease solution (PBS, pH 8 0.25% trypsin; 1 mM EDTA) and incubated for 24 mi at 28°C. During the incubation period, the mixture wa pipetted 50 times every 12 min with a 1-ml pipette tip After all cells were completely separated, 0.2 ml sto solution (30% Fetal bovine serum (FBS); 6 mM $CaCl_2$, PBS) was added, and the mixture was pipetted 50 times and incubated at 28°C for 5 min to stop trypsin activity. Cells were then centrifuged at 3000 rpm for 5 min and re-suspended in 1 ml sorting solution (5% FBS, 50 U/ml penicillin, 0.05 mg/ml streptomycin and PBS). Fluorescence activated cell sorting (FACS) was performed at room temperature under sterile conditions using a FACSAria cell sorting system (BD Bioscience, CA, USA), according to standard protocols described previously (21). Following sorting, when cell viability was >95%, 5×10^5 GFP-positive cells were isolated and 1300 pg of total RNA were extracted to prepare microarray analysis.



2. 水生生物精子质量评估

- 在水生动物人工繁育及品系选育、精子冷冻保存等研究中，对精子进行质量评估，以预测其受精能力是很必要的。
- 流式细胞术，成为了一种检测精液质量的新平台，能为精子功能研究提供快速、客观、多指标、大通量的检测手段。主要检测精子的质膜完整性、顶体状态、染色质结构完整性以及线粒体功能等方面，该技术在哺乳动物精子质量评价中已有较为广泛的应用。流式细胞仪在水生生物精子质量研究的报道较少。

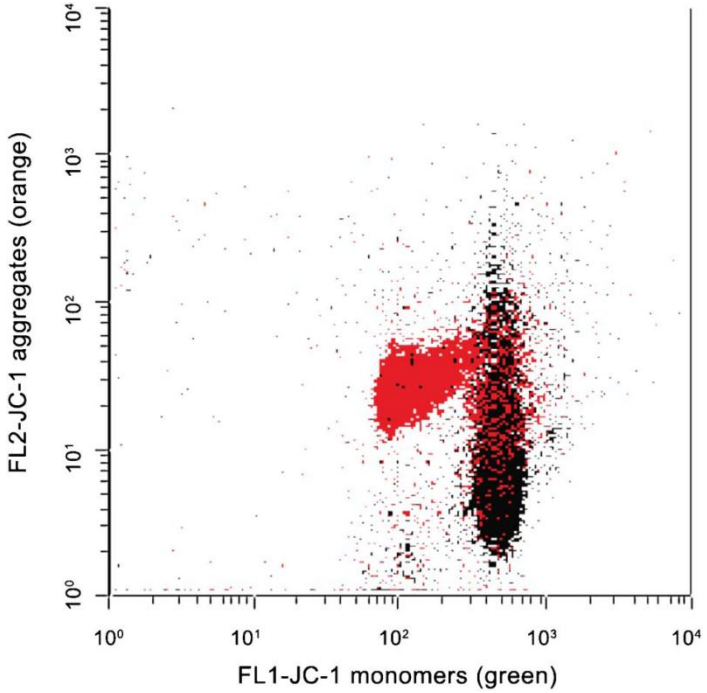


（1）**精子浓度检测：**通过单位体积内收集到的精子数目便可快速地计算出精子的浓度。

（2）**精子质膜的完整性检测：**精子质膜的完整性反映了精子的活力。对精液进行荧光染料染色，根据精子对荧光染料的排斥与否即可鉴定出精子质膜的完整性。常用来检测精子质膜完整性的荧光染料有碘化丙锭(PI)、SYBR系列等。

（3）精子染色质结构的完整性和线粒体功能等。

精子线粒体功能检测——JC-1



荧光染料：

JC-1：阳离子脂质荧光染料

激发波长：488nm

JC-1的变化状态：

A. 低浓度时，以单体存在，可检测到绿色荧光（FL-1/FITC）；

B. 高浓度时，以多聚体存在，可检测到红色荧光（FL-2/PE）；

C. JC-1浓度的变化,在单体和多聚体之间形成一个可逆的转变过程。

专用检测试剂盒：BD Mitoscreen（JC-1）Cat# 551302

正常细胞：JC-1透过细胞膜进入细胞，以单体的状态聚集在细胞内，正常健康线粒体的膜电位（△y）具有极性,JC-1依赖于△y的极性被迅速摄入线粒体内，浓度增高而在线粒体内形成多聚体,多聚体发射光为红色荧光。

凋亡细胞：细胞发生凋亡时,线粒体跨膜电位被去极化,JC-1从线粒体内释放,红光强度减弱,以单体的形式存在于胞质内发绿色荧光。

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Use of JC-1 to assess mitochondrial membrane potential in sea urchin sperm

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[b] Ecotox Services Australasia, 27/2 Chaplin Drive, Lane Cove, NSW 2066, Australia
[c] Marine Ecology Group, Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

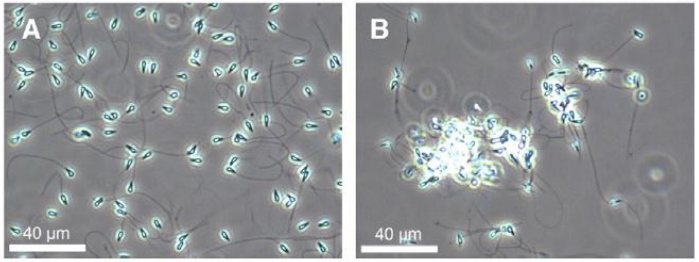

Fig. 2. *C. rodgersii* sperm (A) without JC-1; and (B) after 15-min incubation with 1 µM JC-1.

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Stain

ABSTRACT

There is a need within marine research areas for more rapid techniques to assess the health of sperm from marine invertebrates. Originating in medical research, flow cytometry has been applied to rapidly mea… lular processes within a plethora of different cell types. To date, the transfer of that knowledge … has been limited. A method has been developed to assess mitochondrial membrane potentia… chin (*Centrostephanus rodgersii*) sperm using the stain 5,5′,6,6′-tetrachloro-1,1′3,3′-tetrath… carbocyanine iodide (JC-1) and flow cytometry. MMP is a useful indicator of sperm health as … is the single source of ATP production, and the driver of apoptosis. The method was carefully … idated with the use of positive controls. There were strong correlations between MMP measur… swimming speed and motility (R values of 0.8–0.9, $p < 0.001$). JC-1 successfully differentiat… with low and high MMP. However, in sperm that were treated with the mitochondrial … cyanide 3-chlorophenylhydrazone (CCCP), JC-1 fluorescence in stained sperm did not confo… seen for other cell types. Using fluorescence microscopy, it was confirmed that this was due … of J-aggregates in the acrosome vesicle following MMP collapse. To our best knowledge, this … of J-aggregates forming in an organelle other than the mitochondria. This unexpected fluores… cessitated the use of a quadrant approach (% high MMP) instead of the usual ratiometric app… quantify MMP changes. Difficulties overcome during method development are described, m… likely related to the required use of seawater as a test medium. The developed method will ena… ment of mitochondrial membrane potential of sea urchin sperm for application in reproductiv… ture research, and the impact of environmental stressors such as ocean acidification and p… development and function.

The developed method will enable rapid measurement of mitochondrial membrane potential of sea urchin sperm for application in r**eproductive biology, aquaculture research, and the impact of environmental stressors** such as ocean acidification and pollution on sperm development and function.

应用举例：海胆精子线粒体膜电位检测

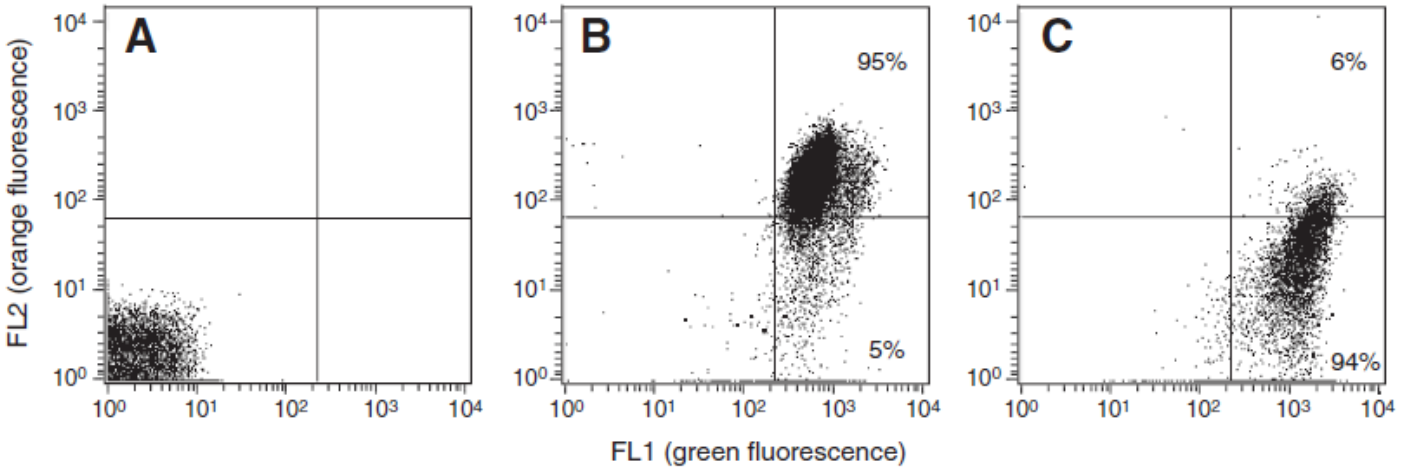


Fig. 1. Representative cytograms showing the fluorescence response for (A) unstained sperm; (B) healthy sperm stained with JC-1 (high MMP); and (C) unhealthy sperm stained with JC-1 (low MMP). The % values represent the proportion of sperm with high MMP (top right quadrant) and low MMP (bottom right quadrant).

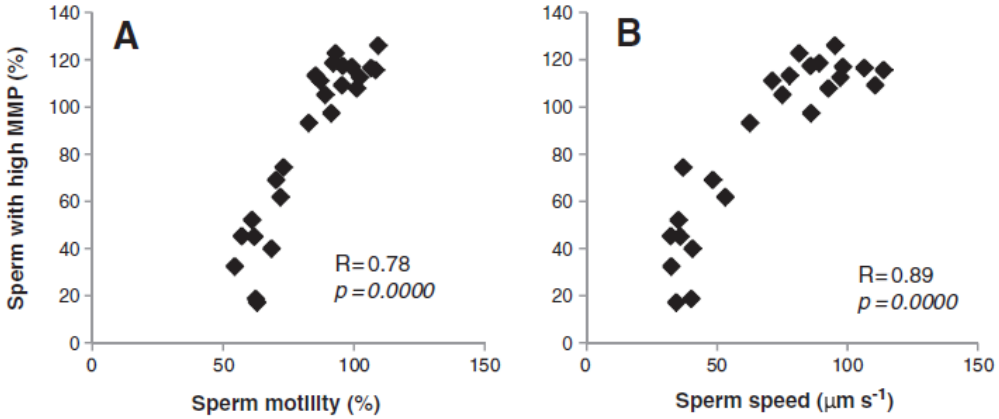


Fig. 7. Relationship between MMP measurements and (A) sperm motility; and (B) sperm speed for *C. rodgersii* sperm following exposure to CCCP (0–4 μM).

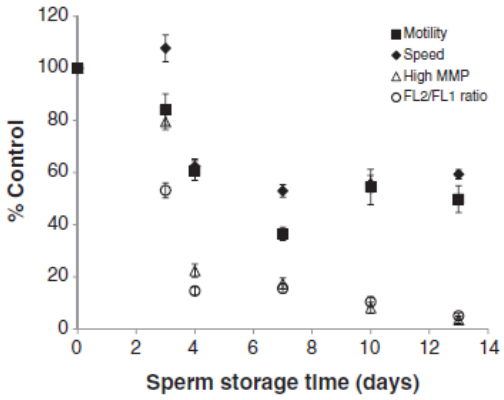


Fig. 8. Motility, speed and MMP measurements of *C. rodgersii* sperm following 15-min incubation with JC-1, over 13 days' storage. Values are means ±1 SE.

主要内容

水体微型生物

水生动物

环境毒理

环境毒理研究

- **环境毒理学研究的发展：**

从经典的单物种实验逐步发展为多物种等模拟生态系统，不仅研究环境污染物对某一种群的危害，而且研究其对生态平衡的影响。

- **研究方法：**

A.从传统的动物体内实验逐步向体外细胞实验发展；

B.从生态水平，个体水平，器官水平，细胞水平和分子水平等多个层面

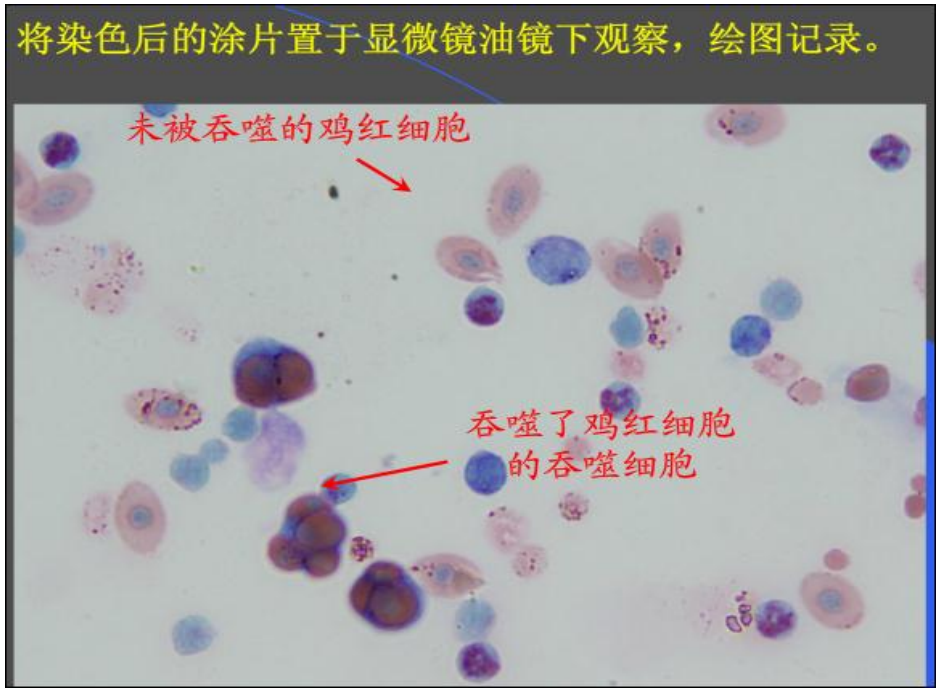
- **研究手段：**

流式细胞术作为单细胞检测的主要技术手段，能够在复杂群体中鉴别群体分类，实时追踪细胞的活性状态，评估细胞的物理和生物学功能等。

因此，流式细胞术在环境毒理学研究一方面体现于**建立一种高效快速的毒性评价方式**，一方面体现于**深入的毒理学机理机制**的研究。

1. 免疫毒理：细胞吞噬实验

- 早期检测方法**以显微观察为主**，如吞噬红细胞实验，白细胞墨汁吞噬实验等



面临问题

- 检测费时费力
- 敏感性低
- 结果易受主观因素影响
- 难以满足大量检验任务要求

流式方法检测细胞吞噬

1982年Steinkamp首创荧光微球流式细胞术定量检测吞噬功能,发展至今,结合各种荧光标记技术,流式细胞术在单核-巨噬细胞吞噬功能研究中的应用很广:

- 吞噬荧光标记细菌；
- 吞噬凋亡细胞
- 吞噬荧光微球；

应用举例：细胞吞噬

Flow cytometric analysis and optimisation for measuring phagocytosis in three Australian freshwater fish

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[a] *Key Centre for Toxicology, School of Medical Sciences, RMIT-University, P.O. Box 71, Plenty Road, Bundoora, Vic. 3083, Australia*

[b] *CENTOX, Manaaki Whenua Landcare Research, P.O. Box 69, Lincoln 8152, New Zealand*

Received 16 November 2004; revised 16 June 2005; accepted 11 July 2005
Available online 26 September 2005

Abstract

The phagocytic activity of fish immunocytes has been measured by a wide range of methods, and has been used as a bio-indicator to assess the immunotoxicity of environmental pollutants and the efficiency of immunostimulants used in aquaculture. This study demonstrates the utilisation of a flow cytometric technique for measuring phagocytosis as an alternative to manual evaluations by light microscopy. Optimal conditions for the phagocytosis of latex beads were ascertained, including incubation period, cell:bead ratio and media components, for head kidney cells isolated from three native Australian fish that inhabit the Murray–Darling basin, i.e. silver perch (*Bidyanus bidyanus*), golden perch (*Macquaria ambigua*) and crimson-spotted rainbowfish (*Melanotaenia fluviatilis*). Thus, standardised protocols have now been established for future use in the immunotoxicity testing of xenobiotics in native Australian freshwater fish.

应用举例：细胞吞噬

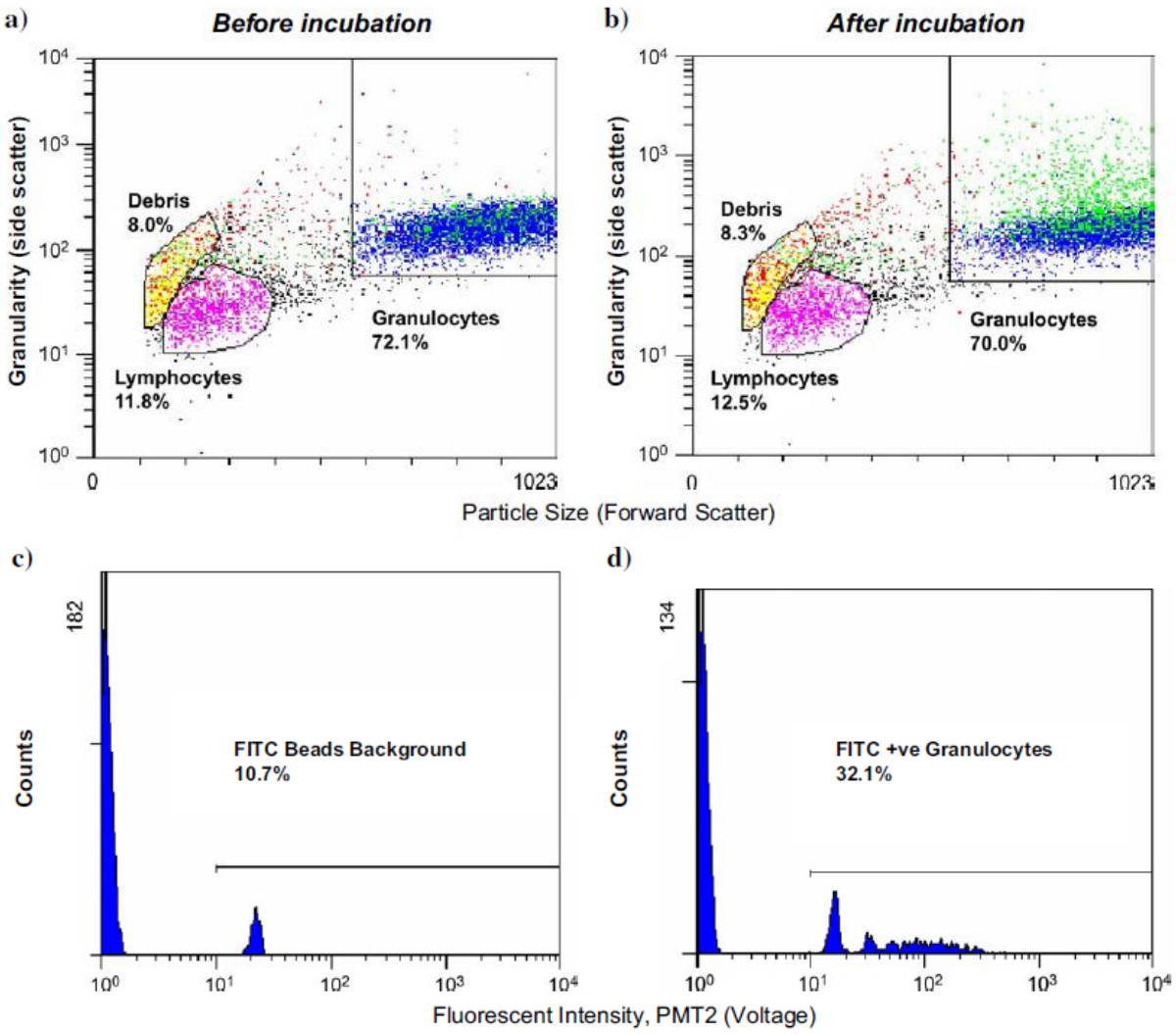


Fig. 1. Typical FS vs. SS plots of native fish h head kidney cells,

(a) before (negative control)

(b) after incubation with FITC-latex beads. The FITC 'signal' measured in PMT 2 shows

(c) negative control with a single peak representing unengulfed beads

(d) multiple peaks after incubation with beads, representing phagocytes that have internalised multiple beads. Colours representation:granulocytes, blue; lymphocytes, pink; debris, yellow; FITC-positive, green; PI-positive, red.

免疫毒理：活性氧簇（ROS）检测

- **呼吸爆发活力**常作为反映水生生物免疫状况的重要指标。

- **原理**：当病菌侵入机体时，具免疫功能的细胞通过吞噬和杀菌作用杀灭病原，在吞噬杀菌过程中产生大量还原性物质，如H_2O_2 、超氧阴离子(0^-_2)等活性氧簇（ROS），这一过程称为“呼吸爆发”。

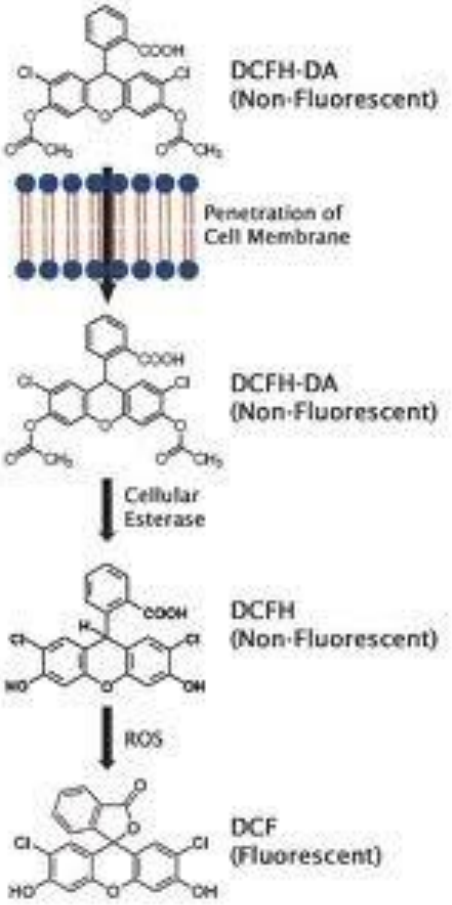
- **应用**：受外界胁迫环境的诱导，盐度、pH、温度、重金属等环境应激均会导致ROS的大量上升，因此ROS 含量也是反映水生生物是否受到不良因素胁迫的重要指标，可作为环境污染物胁迫的评价指标。

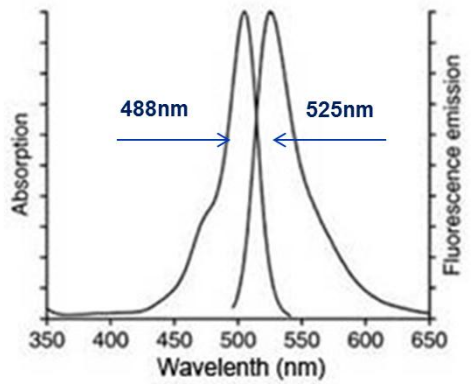
- **检测手段**：ROS 在生物体内的寿命很短，建立一种更加快速、灵敏的ROS 检测方法尤为重要。

流式检测方法：

- DCFH-DA：2′,7′-二氢二氯荧光黄双乙酸钠
- 检测原理：DCFH-DA，活性氧的特异探针，它本身不发荧光，可自由穿过细胞膜进入到细胞内，被胞内的酯酶分解为DCFH 而保留在胞内，各类ROS会氧化DCFH 为发强绿色荧光的DCF，DCF被488nm激光激发，发射530nm左右的荧光信号，利用流式细胞仪检测胞内的DCF 荧光强度即可反映细胞的ROS 水平。

- DHR ：双氢罗丹明
- 检测原理：可自由进出细胞膜，进入细胞后可被氧化为可发出荧光的罗丹明-123，经过一定时间的积累，即可通过流式细胞仪检出相应的荧光。





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PLOS ONE

Phagocytosis and Respiratory Burst Activity in Lumpsucker (*Cyclopterus lumpus* L.) Leucocytes Analysed by Flow Cytometry

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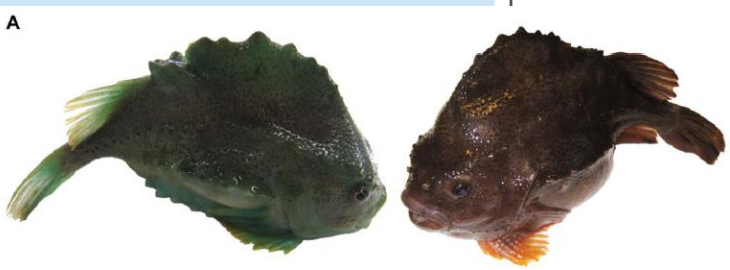
Abstract

In the present study, we have isolated leucocytes from peripheral blood, head kidney and spleen from lumpsucker (*Cyclopterus lumpus* L.), and performed functional studies like phagocytosis and respiratory burst, as well as morphological and cytochemical analyses. Different leucocytes were identified, such as lymphocytes, monocytes/macrophages and polymorphonuclear cells with bean shaped or bilobed nuclei. In addition, cells with similar morphology as described for dendritic cells in trout were abundant among the isolated leucocytes. Flow cytometry was successfully used for measuring phagocytosis and respiratory burst activity. The phagocytic capacity and ability were very high, and cells with different morphology in all three leucocyte preparations phagocytised beads rapidly. Due to lack of available cell markers, the identity of the phagocytic cells could not be determined. The potent non-specific phagocytosis was in accordance with a high number of cells positive for myeloperoxidase, an enzyme involved in oxygen-dependent killing mechanism present in phagocytic cells. Further, high respiratory burst activity was present in the leucocytes samples, verifying a potent oxygen-dependent degradation. At present, the specific antibody immune response could not be measured, as immunoglobulin or B-cells have not yet been isolated. Therefore, analyses of the specific immune response in this fish species await further clarification. The present study presents the first analyses of lumpsucker immunity and also the first within the order Scopaeniformes.

圆鳍鱼外周血，头肾和脾脏白细胞吞噬能力和呼吸爆发的评估



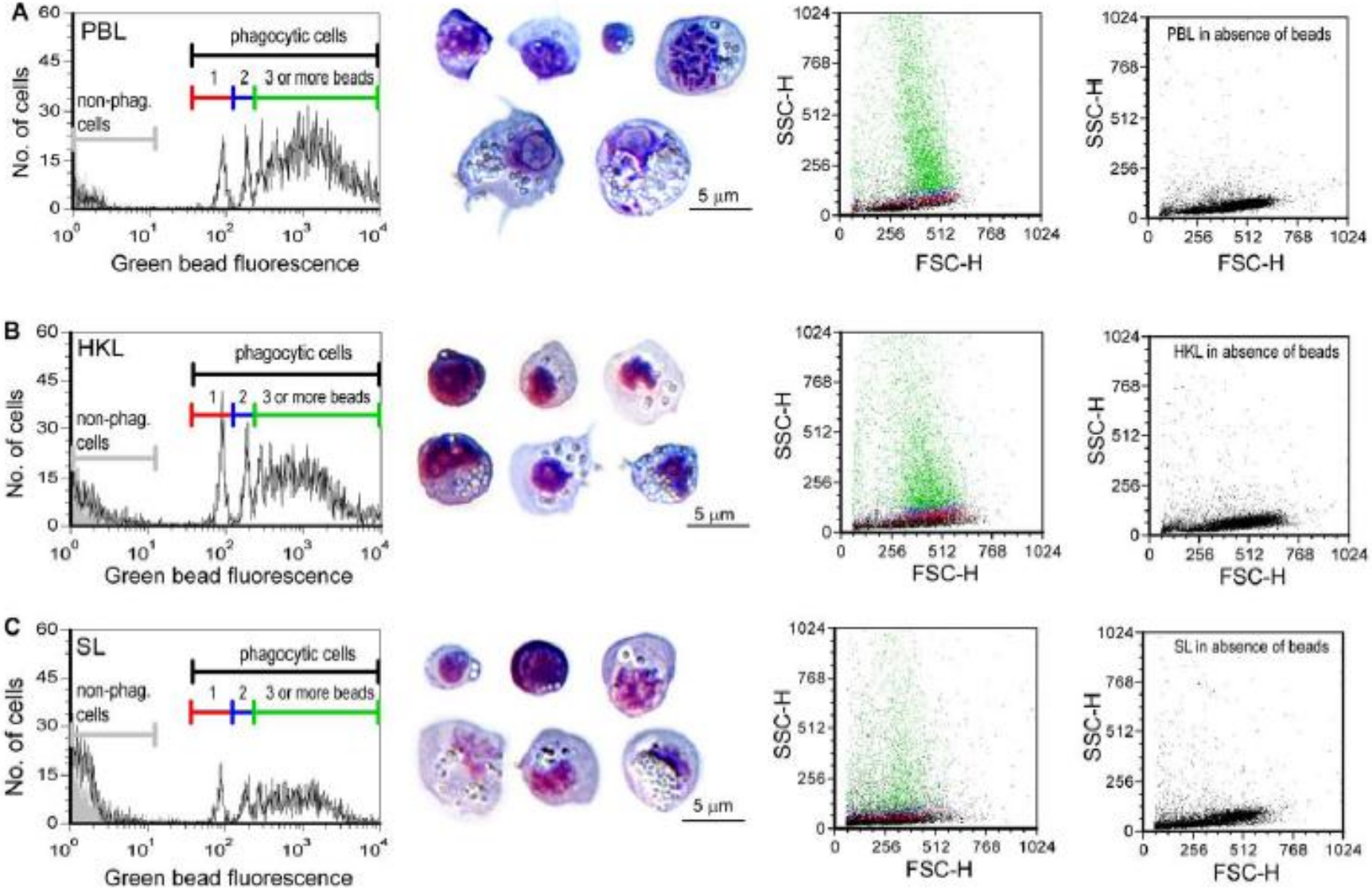


Figure 5. The phagocytic capacity of isolated leucocytes is high. FL1 (green bead fluorescence) histograms (left) showing phagocytic capacity of PBL (A), HKL (B) and SL (C) incubated with fluorescent beads (1 µm) for 4 h. Increased peak fluorescence indicates an increased number of ingested beads. Picture insets show cells stained with Colorrapid from PBL, HKL and SL samples that have ingested various numbers of beads. The left dot plots show cells in the red (cells with 1 bead) blue (cells with two beads) and green (cells with 3 or more beads) and black (non-phagocytic cells) regions; cells with a higher number of ingested beads have a higher granularity (SSC-value). The dot plots to the right show the light scatter properties of the cells incubated without beads at the instrument settings used for the phagocytosis assay.

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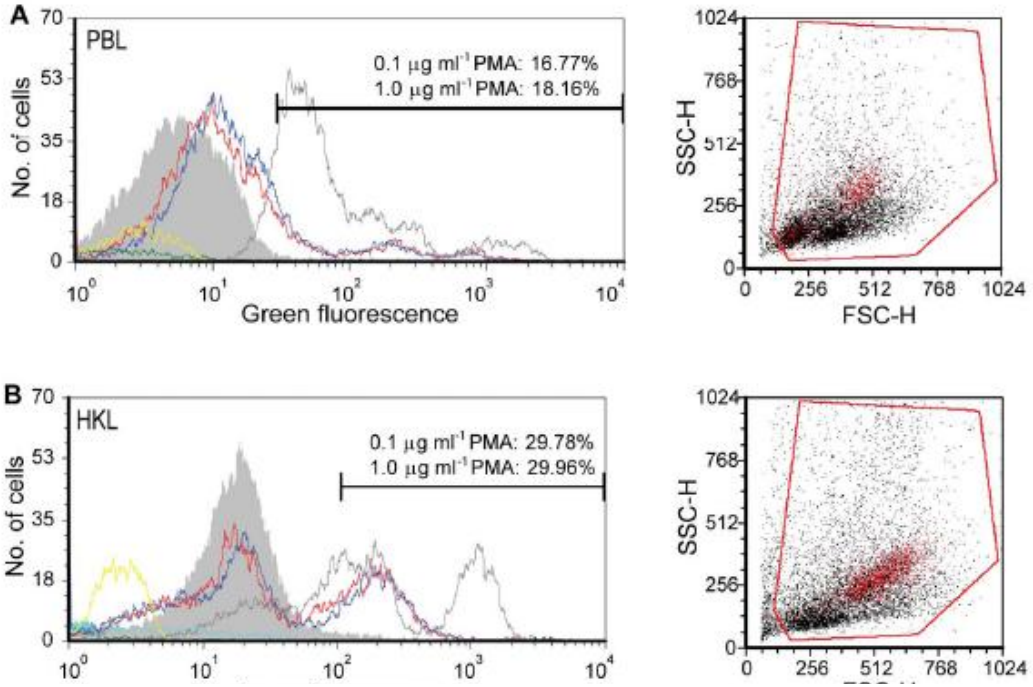

Table 1. The proportions of RHO-positive cells and geometric mean fluor
SL from lumpsucker analysed by flow cytometry.

	PBL[a]		HKL[b]
PMA (µg ml^{-1})	% RHO-pos	GMFI	% RHO-pos
–	2.0	12.2±2.7	2.0
0.1	16.2±9.8	25.9±16.7	18.8±6.6
1.0	17.4±11.1	27.1±18.1	24.2±6.9

[a]N = 4, [b]N = 5

doi:10.1371/journal.pone.0047909.t001

Flow Cytometry Assay of Respiratory Burst

The flow cytometry analyses of respiratory burst is based on previous established protocols for measurements of respiratory burst in cod and salmon where dihydrorhodamine 123 (DHR) is oxidised to the fluorescent rhodamine 123 (RHO) [28]. From a leucocyte concentration of 2.5×10^6 ml^{-1}, 200 µl was transferred to 5 ml polystyrene tube (Falcon, Becton Dickinson, Franklin lakes, USA) and incubated at 18°C for 10 min with gentle tilting. Respiratory burst was activated in leucocytes by PMA (Sigma, St. Louis, USA) using a concentration of 0.1 and 1 µg ml^{-1} PMA in the tube and incubated for 10 min at 18°C before addition of DHR. The PMA stock solution was made of 1 mg PMA in 1 ml DMSO $(CH_3)_2SO$, (Sigma, St. Louis, USA) and was stored at −20°C and further diluted in PBS 380 containing heparin (PBS 380 h). Five µl of 206 µM DHR, resulting in a total concentration of 5 µM per sample tube was added. The samples were mixed and incubated by gentle tilting for 15 min. Prior to flow cytometry analyses 300 µl PBS 380 h was added to each tube and the cells were carefully suspended using a vortex mixer. Two parallels per variable from 5 fish were used throughout all analyses.

The flow cytometry analyses and data analyses were performed as described above. RHO fluorescence was detected with 530/30 nm bandpass filter; FL1.

Several controls were included in the respiratory burst analyses. Untreated leucocytes control without PMA and DHR for detection of any possible auto fluorescence from the leucocytes. A PMA stimulated samples without DHR were analysed to test for presence of fluorescence not caused by oxidation of DHR. A non-

2. 水生态毒理

- 研究水体中种群生长抑制率是水体环境毒性实验的关键指标之一，传统的方法，如镜检法和光密度法等，检测灵敏度低，而且不能有效地区分水体中非藻类，细胞碎片以及死细胞，种群异质性等的影响。

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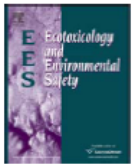

Toxicity and bioaccumulation of copper and lead in five marine microalgae

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ABSTRACT

On five marine microalgae with the same biovolume quantity (*Tetraselmis chuii*, *Rhodomonas salina*, *Chaetoceros* sp., *Isochrysis galbana* (T-iso) and *Nannochloropsis gaditana*) 72-h exposu with copper and lead were performed. For both metals, 72-h EC50s showed *T. chuii* as t and *R. salina* as one of the most sensitive. Besides copper and lead EC_{50} conce concentrations in solution and accumulated on/in the cell where also analysed. *T.* tolerant species accumulated high copper concentrations ($EC_{50(Cu)} = 330\,\mu g\,L^{-1}$; E L^{-1}), and *R. salina* the most sensitive to copper, accumulated the highest amou ($EC_{50(Cu)} = 50\,\mu g\,L^{-1}$). Results of this study show that there is no specific relationsh tolerance and accumulated metal on/in the cell. On the other hand, due to an establi the influence of cellular density in microalgae toxicity tests, this effect was also studied reduced EC_{50} values when initial cellular densities decreased.

In this study, the term "toxic cellular quota" was used to express all data. This all expression, the combination of two parameters that clearly influence growth, cellular concentration.

2.5. Flow cytometry analysis

Samples for analysis by flow cytometry were collected from the toxicity tests after 72 h of treatment. These were analysed using a FACScalibur flow cytometer equipped with a 488 nm excitation argon laser and the data were computed with CellQuest software (Beckton-Dickinson). Each culture was immediately analysed for 30–60 s (6000–10,000 events per measurement) from samples previously fixed with 3–4% formaldehyde. Counts, signals of side-angle light scatter (SSC), and autofluorescence (FL3, >630 nm) were recorded and used as indicators of the cellular size and chlorophyll fluorescence, respectively (Sobrino et al., 2004).

通过藻类色素自发荧光，利用流式细胞仪分析了重金属离子铜和铅对5种微藻的生长抑制的影响。

应用举例：水生态毒理

Determination of short-term copper toxicity in a multispecies microalgal population using flow cytometry

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Available online 20 December 2005

Abstract

This study was conducted to determine the role of algal–algal interactions in a multispecies microalgal to copper based on an enzyme inhibition assay using flow cytometric measures. Autofluorescence (chlo used to identify species and count algal signals. The effect of multispecies population on copper toxicity detected (1) at the same initial cell density, (2) at the same surface area, and (3) in the presence and abse *Scenedesmus obliquus*. As copper concentrations increased, esterase activity of *M. aeruginosa* change manner. The 24 h EC_{50} value of *M. aeruginosa* in the multispecies population was significantly ($P<0.05$ species population. Compared with *S. obliquus*, the effect of *C. pyrenoidosa* on *M. aeruginosa* was more copper on fluorescin diacetate fluorescence of *M. aeruginosa* was 11 μg/L). At 48 h copper exposure (reactive oxygen species levels also showed similar algal–algal interactions in multispecies microalgal suggested that these algal–algal interactions occurred only at low concentrations (<13 μg/L, 24 and 48 demonstrates the importance of using multispecies populations to estimate metal toxicity in natural wa

Keywords: Copper; Multispecies microalgal population; Toxicity; Algal–algal interactions; Flow cytometry

南京中科院地理湖泊研究所发表的这篇文章中，展示了利用流式细胞术进行多种群的毒理学研究方法，通篇文章80%的实验工作都是基于流式分析完成，包括藻类分群、计数、脂酶活性检测、ROS水平检测。

2.4. Flow cytometric analysis

Flow cytometric analysis was carried out with a FACSVantage SE flow cytometer (Becton–Dickinson, USA) equipped with a dual-laser bench and optics (Coherent Innova 70-4 laser emitting at 488 nm; fluorescence was collected at a range of wavelengths by three-colored photomultiplier tubes with fluorescence emission filters (FL1 530/30 nm, FDA fluorescence or H_2DCFDA fluorescence; FL2 580/42 nm; and FL3 675/20 nm, Chl *a* fluorescence); Coherent 599 dye laser emitting at 600 nm, bumped by a Coherent Innova 305 laser; fluorescence was collected at 660/20 nm (FL4, PC fluorescence). Nonalgal particles and dead cells were excluded from the analysis by gating on FSC/FL3/FL4. Flow rate of flow cytometry was 1 μL/s. Acquisition was made using the pulse height and log mode for all variables. The program Cell-Quest from Becton–Dickinson was used to collect and analyze these signals.

Caltag counting beads (Caltag Laboratories, Inc., CA) were used to calibrate the algal cell density counts in each species. A known volume of Caltag counting beads (A and B beads) was added to the same volume of cells. As the concentration of beads was known, the number of cells per microliter (the absolute count) can be determined by relating the number of cells counted to the total number of fluorescent bead events. As the Caltag counting beads system contained two different fluorospheres of known proportions, the accuracy of the assay could be assured by verifying the proportions of both types of beads. The final absolute count was determined as described in the following process:

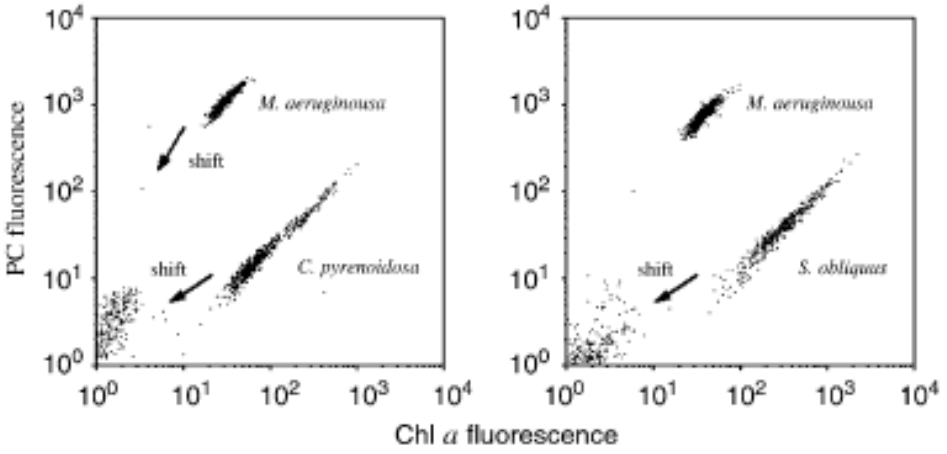


Fig. 1. Two-dimensional cytogram of Chl *a* fluorescence and PC fluorescence showing species separation for multispecies bioassays (*M. aeruginousa* vs *C. pyrenoidosa* and *M. aeruginousa* vs *S. obliquus*). Arrows show the shifts of algae after copper exposure.

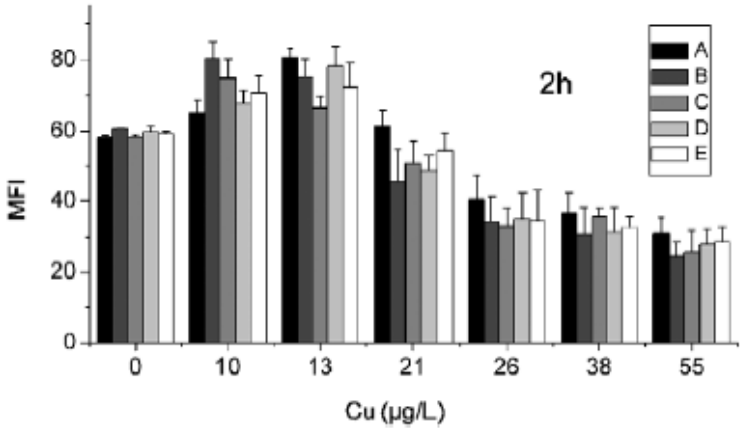


Fig. 2. Mean fluorescence intensity (MFI) of *M. aeruginousa* exposed to copper for 2 h in single- and multispecies bioassays. Data points represent the mean ± standard error of the mean ($n = 5$). (A) Single species, initial cell inocula: 4.0×10^4; (B) single species, initial cell inocula: 2.0×10^4; (C) multispecies, initial cell inocula: 2×10^4, with *C. pyrenoidosa* (2.0×10^4); (D) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (0.9×10^4); (E) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (2.0×10^4).

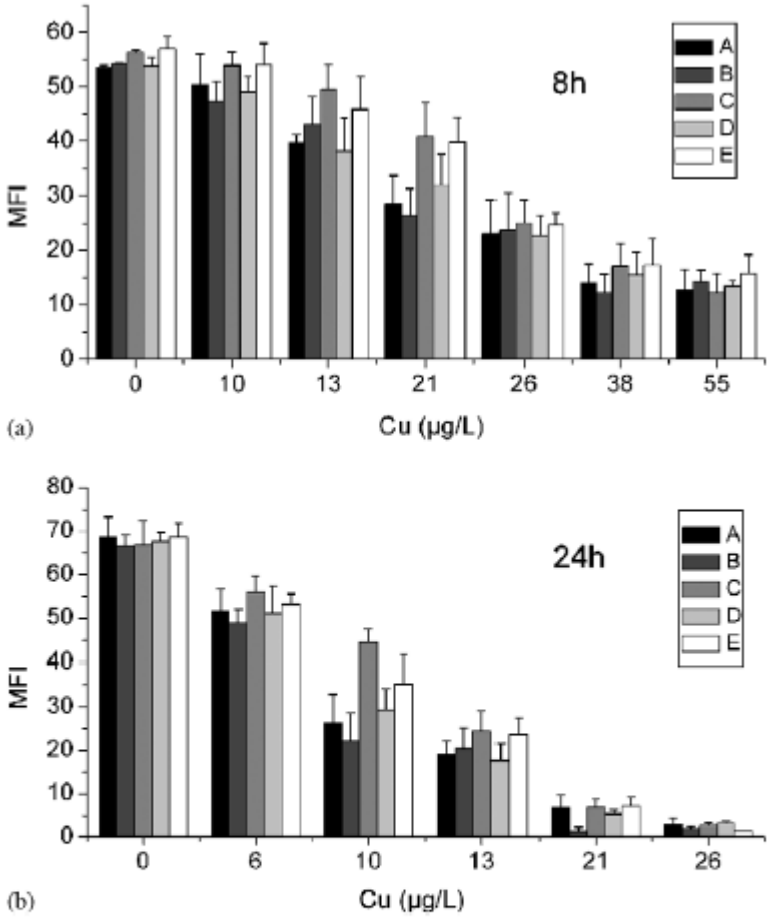


Fig. 3. Mean fluorescence intensity (MFI) of *M. aeruginousa* exposed to copper for 8 and 24 h in single- and multispecies bioassays. Data points represent the mean ± standard error of the mean. ($n = 5$). (A) Single species, initial cell inocula: 4.0×10^4; (B) single species, initial cell inocula: 2.0×10^4; (C) multispecies, initial cell inocula: 2×10^4, with *C. pyrenoidosa* (2.0×10^4); (D) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (0.9×10^4); (E) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (2.0×10^4).

3.3. Changes in ROS levels of cells

To investigate these algal–algal interactions further, the intracellular ROS level in each group was also tested at a low copper concentration. As shown in Fig. 4, exposure of *M. aeruginosa* cells to copper (6 μg/L) induced time-dependent increases in ROS formation, as measured by the oxidation-sensitive indicator H_2DCFDA. Mainly, these increases appeared after 48 h exposure. At this time, the ROS level of *M. aeruginosa* in the single-species populations (groups A, B) were significantly higher ($P < 0.05$) than those in the multispecies populations (groups C–E) either at the same initial cell densities or on similar surface areas.

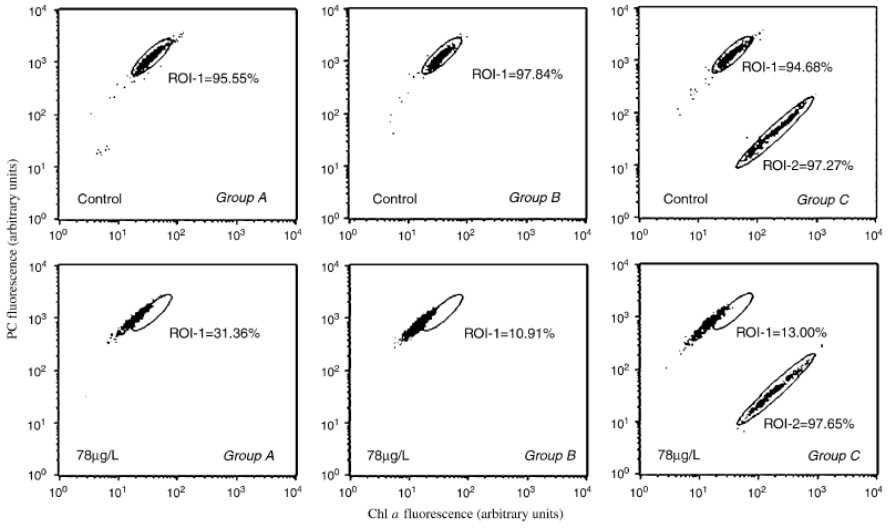


Fig. 5. Flow cytometry dotplot showing shifts in autofluorescence (Chl *a* vs PC fluorescence) of *M. aeruginousa* and *C. pyrenoidosa* cells after a 24-h Cu exposure (FL3, Chl *a* fluorescence; FL4, PC fluorescence). ROI, region of interest. ROI-1 and ROI-2 represent the regions of *M. aeruginousa* and *C. pyrenoidosa* cells, respectively. Group A, single species, initial cell inocula: 4.0×10^4; group B, single species, initial cell inocula: 2.0×10^4; group C, multispecies, initial cell inocula: 2.0×10^4, with *C. pyrenoidosa* (2×10^4).

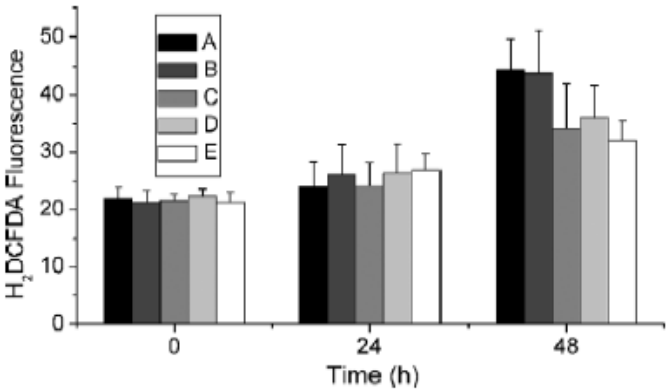


Fig. 4. Time course of ROS production induced by Cu in *M. aeruginosa* cells. Data points represent the mean ± standard error of the mean ($n = 3$). (A) Single species, initial cell inocula: 4.0×10^4; (B) single species, initial cell inocula: 2.0×10^4; (C) multispecies, initial cell inocula: 2×10^4; with *C. pyrenoidosa* (2.0×10^4); (D) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (0.9×10^4); (E) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (2.0×10^4).

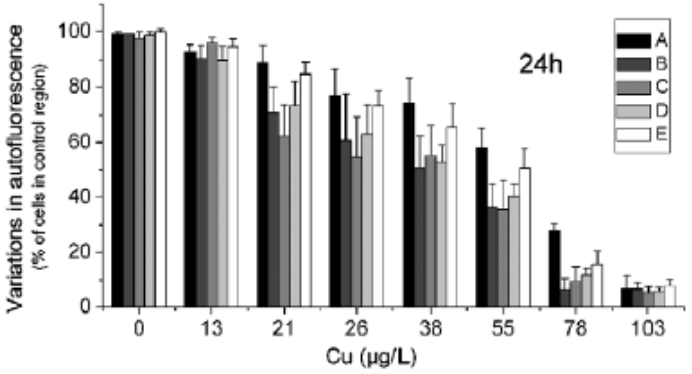
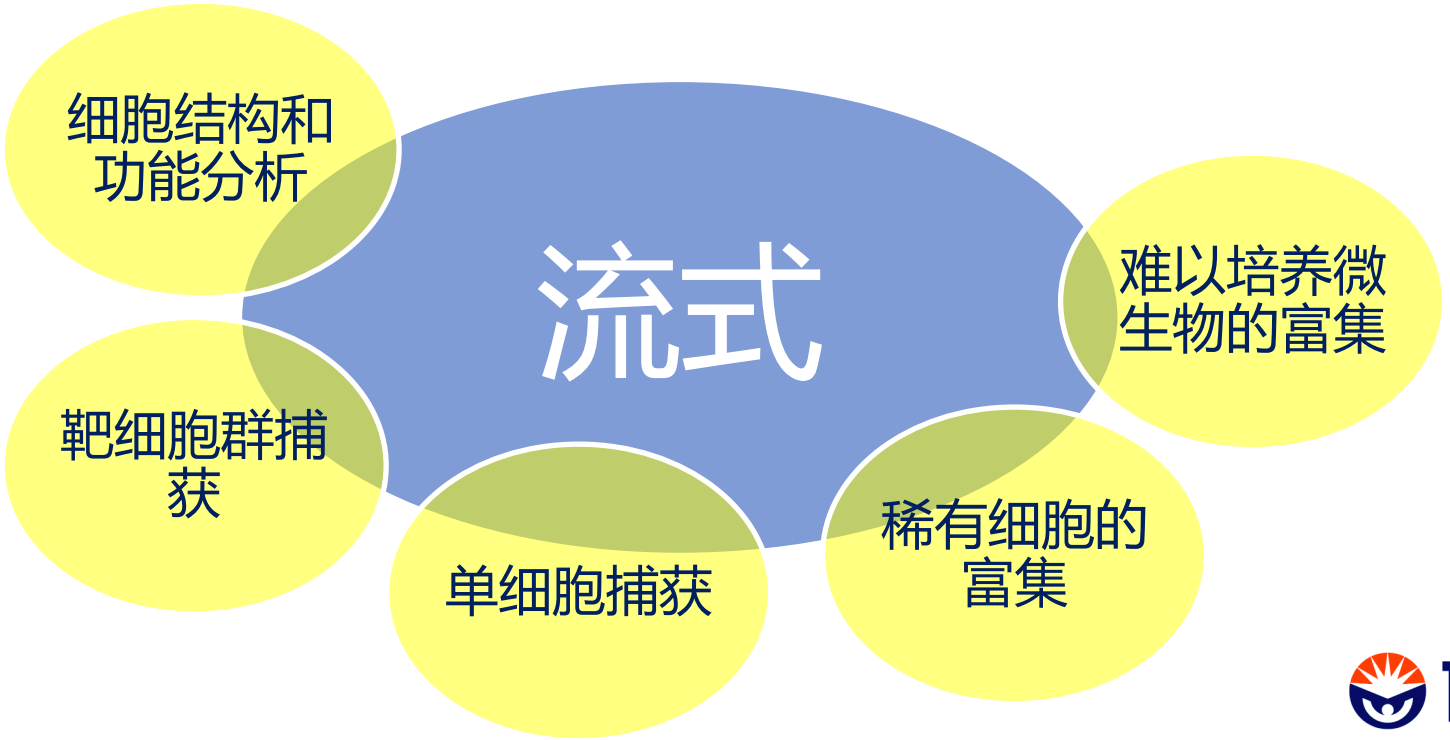


Fig. 6. Shifts in autofluorescence (Chl *a* vs PC fluorescence) of *M. aeruginousa* cells after a 24-h Cu exposure. (A) Single species, initial cell inocula: 4.0×10^4; (B) single species, initial cell inocula: 2.0×10^4; (C) multispecies, initial cell inocula: 2×10^4, with *C. pyrenoidosa* (2.0×10^4); (D) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (0.9×10^4); (E) multispecies, initial cell inocula: 2×10^4, with *S. obliquus* (2.0×10^4).

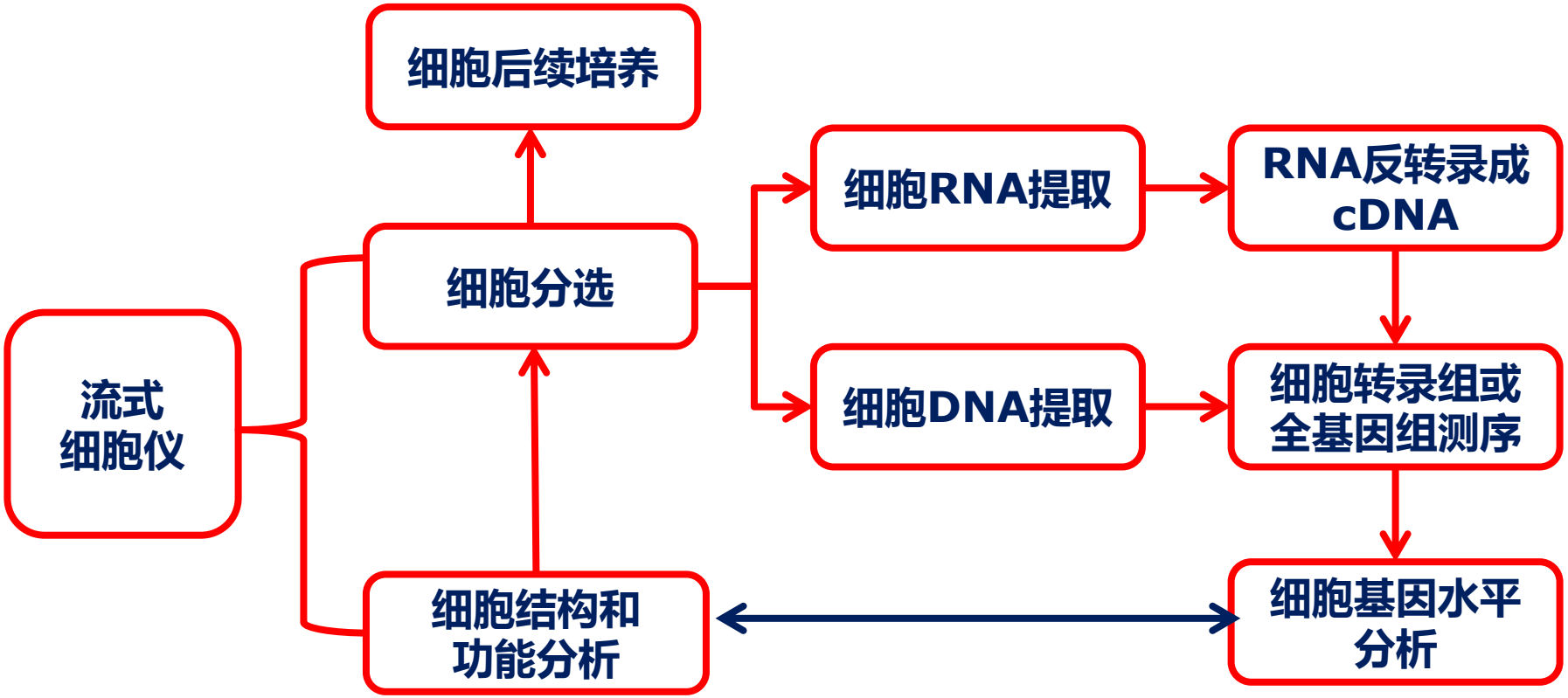
all people
thy lives

总结

- 流式分析：揭示细胞结构和功能
- 流式分选：靶细胞群分离和单细胞分离的主流技术
- **流式细胞术：将细胞的生命状态和基因表达调控有机结合**



关于细胞的完整解决方案



BD

Helping all people live healthy lives

Thanks !

Q&A

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