



Agenzia nazionale per le nuove tecnologie,
l'energia e lo sviluppo economico sostenibile

Citometria a flusso: quale contributo in ambito vegetale? Alcuni esempi

Debora Giorgi, ENEA - Roma

Il primo articolo relativo all'analisi di cellule vegetali mediante Citometria a Flusso (CF) risale al 1973 ma solo negli anni 80 l'applicazione di questa tecnica ha visto un crescente impiego, grazie anche alla metodica rapida e semplice di estrazione dei nuclei da tessuti vegetali, messa a punto da Galbraith (1983). Oggi il notevole sviluppo tecnologico degli strumenti di analisi, accanto alla messa a punto di protocolli innovativi e di nuovi connubi tra la citometria e altre tecniche di indagine molecolare, stanno portando ad un ampliamento dei settori di applicazione della CF a supporto di studi tassonomici, evolutivi, genomici etc... e per diversi obiettivi come il *plant breeding* e il sequenziamento dei genomi. Verranno presentati alcuni esempi di applicazioni in ambito vegetale, con particolare riferimento all'analisi di nuclei, per la determinazione del contenuto di DNA e del livello di ploidia per identificare poliploidi naturali ed artificiali e tracciare in ibridi interspecifici, e saranno discussi i risultati dell'analisi di sospensioni cromosomiche e il *chromosome sorting* in specie appartenenti prevalentemente alla famiglia delle Poaceae (grani). La tecnica di FISH in Sospensione (FISHIS), messa a punto nei nostri laboratori, sarta' presa ad esempio come nuova metodologia in grado di favorire ed estendere le applicazioni della CF consentendo di estendere il *chromosome sorting* a diverse specie, varietà o linee di interesse agronomico, consentendo l'isolamento di cromosomi "alieni".



1101 0110 1100
0101 0010 1101
0001 0110 1110
1101 0010 1101
1111 1010 0000





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Citometria a flusso: quale contributo in ambito vegetale? Alcuni esempi.

Roma 15 aprile 2019

Giorgi Debora



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1101 0010 1101
1111 1010 0000

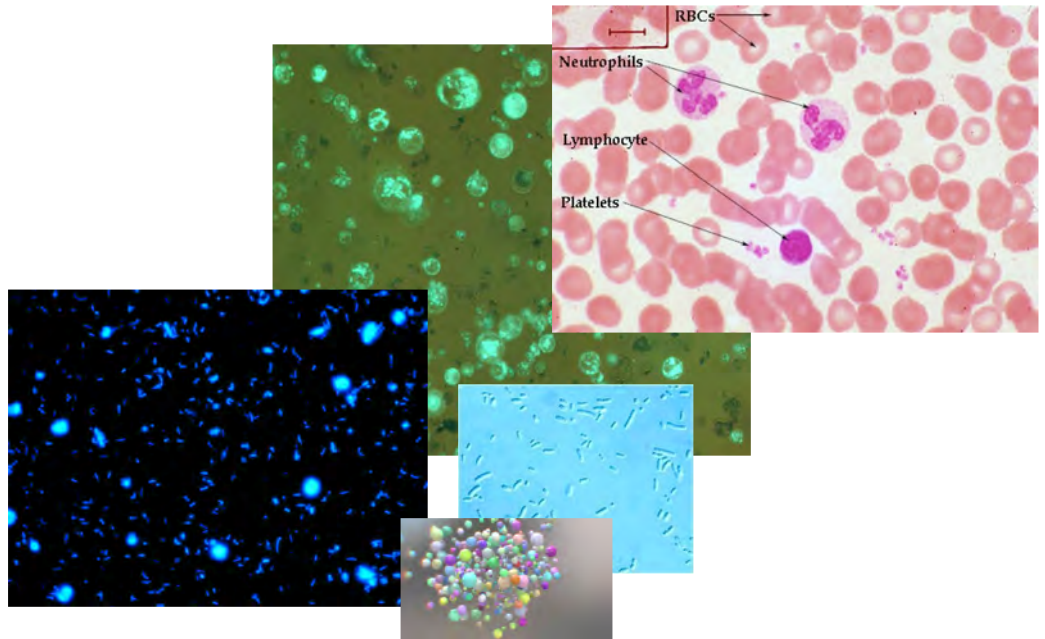


La citometria a Flusso

La Citometria a Flusso (FCM) è una tecnica che consente di analizzare alcune **proprietà ottiche** di **singole cellule/particelle in sospensione**.

VANTAGGI

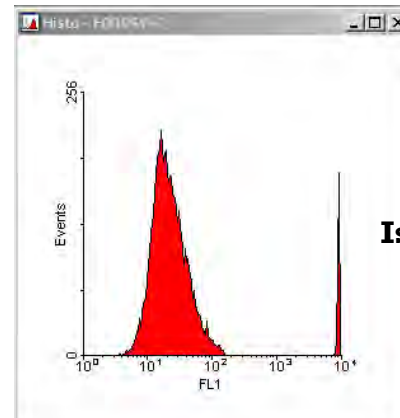
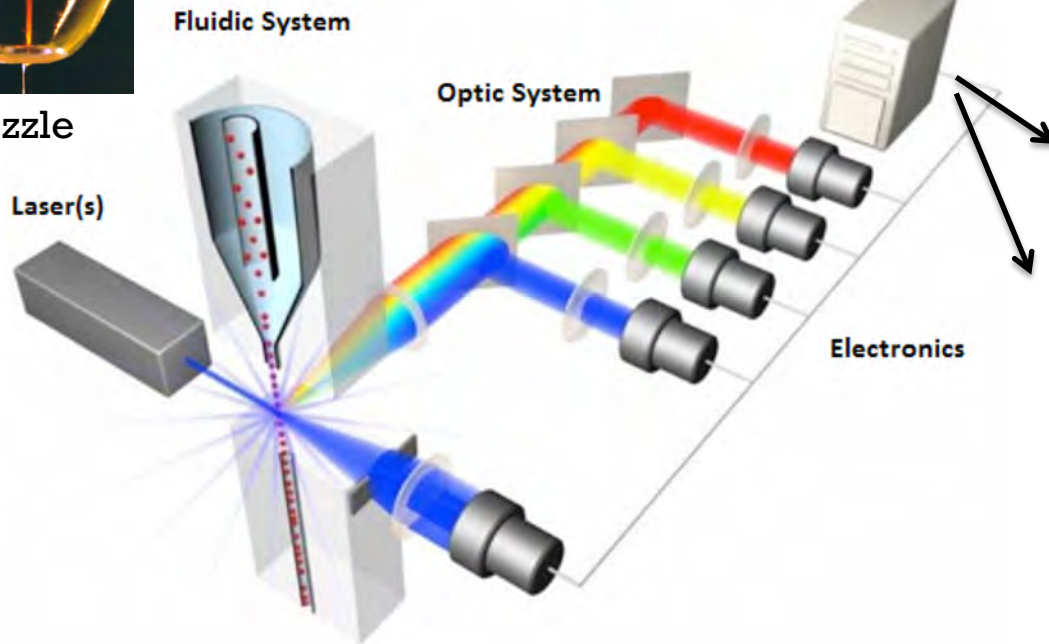
- alto “throughput”
- multiparametricità
- elevata risoluzione
- velocità di analisi
- robusta statistica



Principali componenti di un Citometro a Flusso

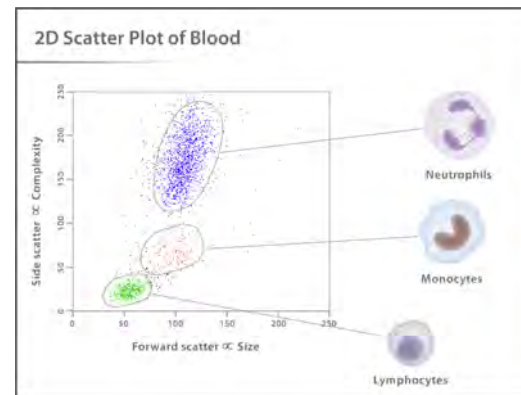


nozzle



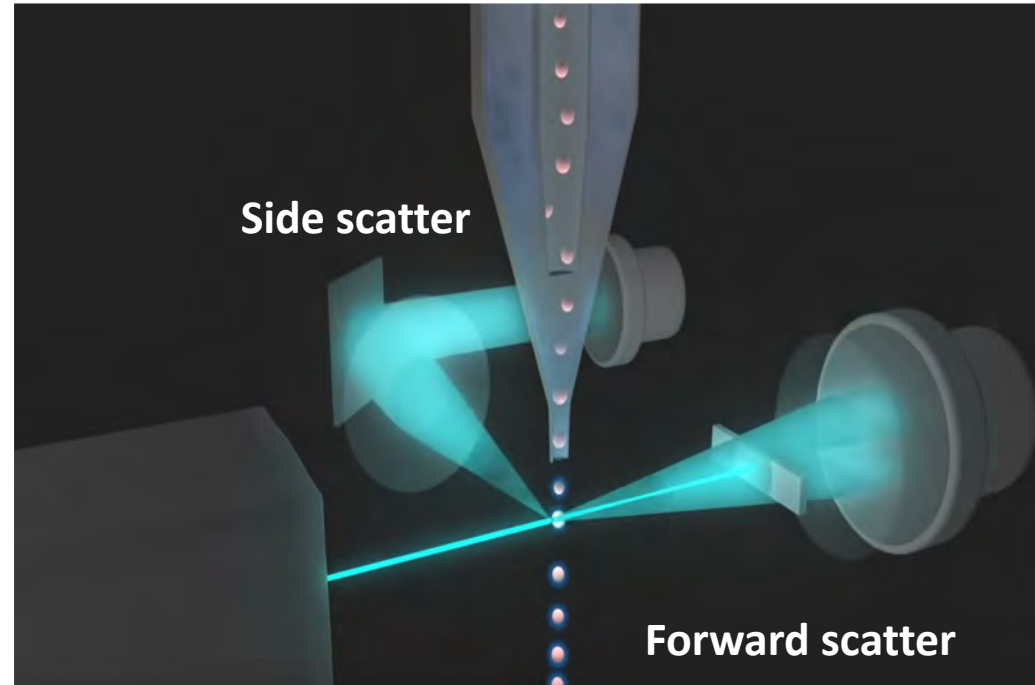
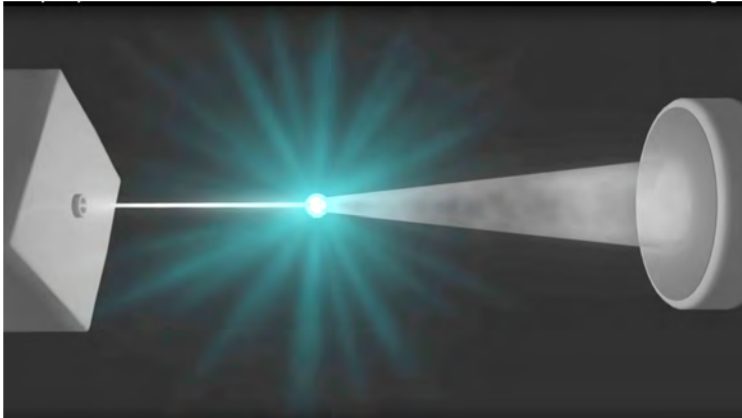
Istogramma

Dot Plot



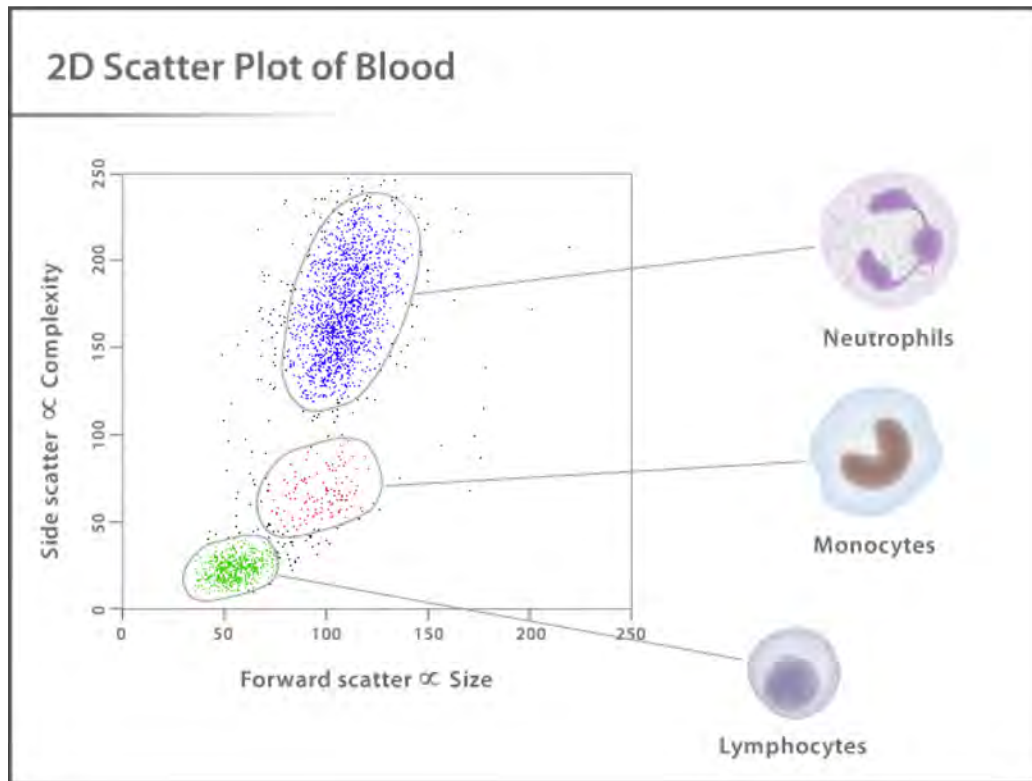
Parametri ottici: light scattering

Dall'interazione luce-particella scaturiscono i segnali di light-scattering

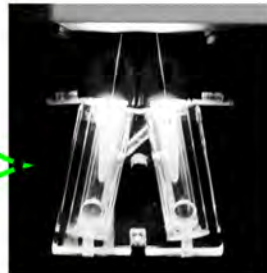
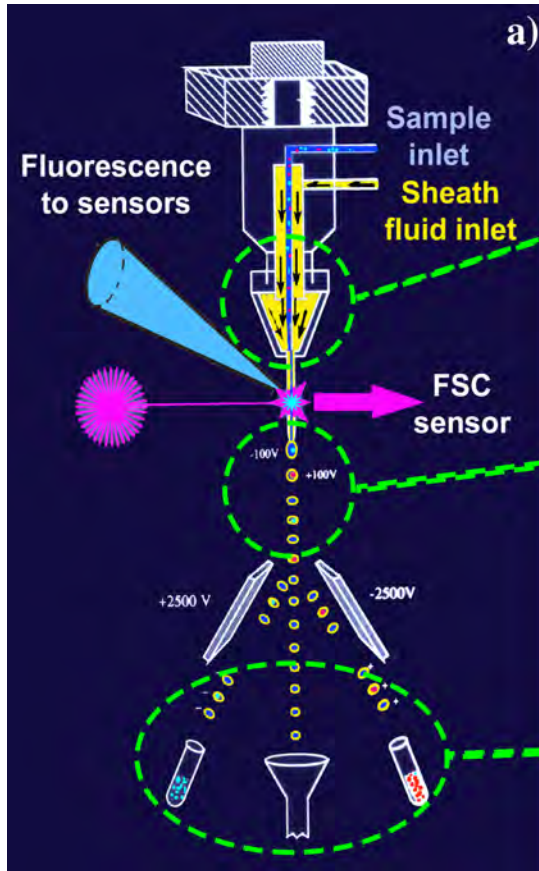


Discriminazione cellule del sangue

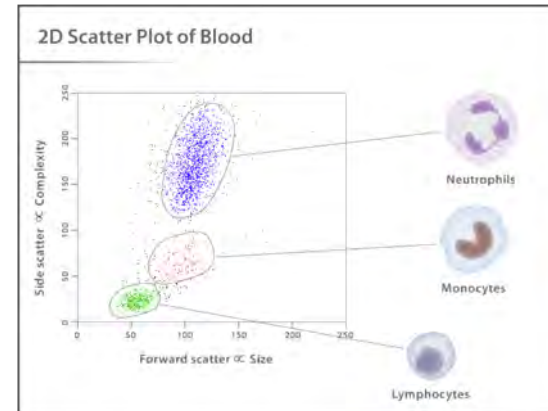
Forward Scatter (FSC) e Side Scatter (SSC)
Consentono di discriminare alcune tipologie di cellule in base a dimensioni (FSC) morfologia e granulosità (SSC)



Flow sorting



Se il citometro è anche un “SORTER” è possibile isolare “sortare” le popolazioni di interesse

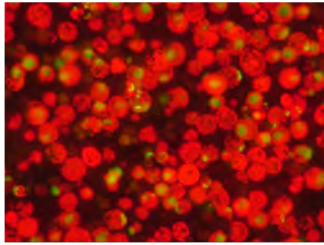


Le particelle selezionate vengono dotate di carica elettrica e deviate dalla traiettoria lineare mediante piastre cariche elettricamente

Parametri ottici: fluorescenza

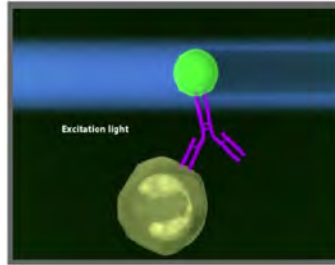
E' possibile marcare in modo specifico alcune tipologie di cellule o alcuni componenti o attività cellulari...

**Fluorescenza naturale
(clorofilla)**

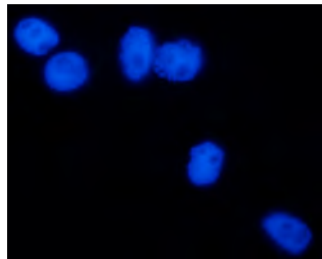


Protoplasti di *citrus* (Clorofilla)

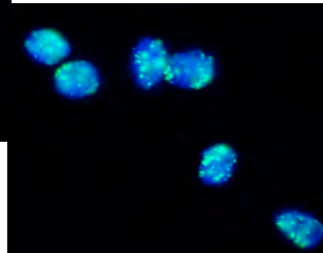
Fluorescenza indotta



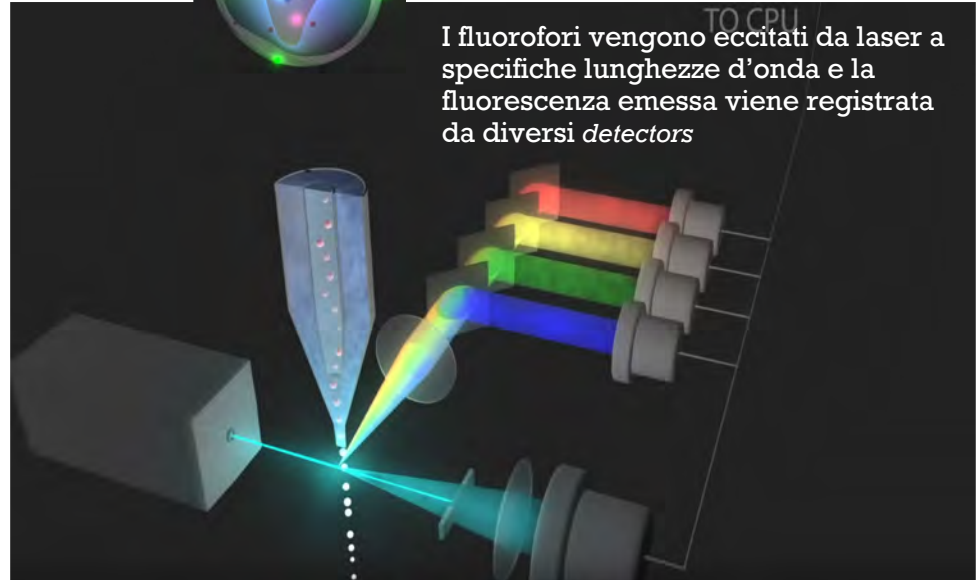
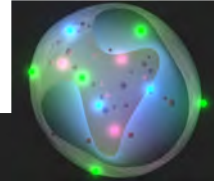
Anticorpo coniugato con
fluoroforo



Nuclei di grano
colorati con DAPI e...



marcati mediante
(GAA)₇FISHIS

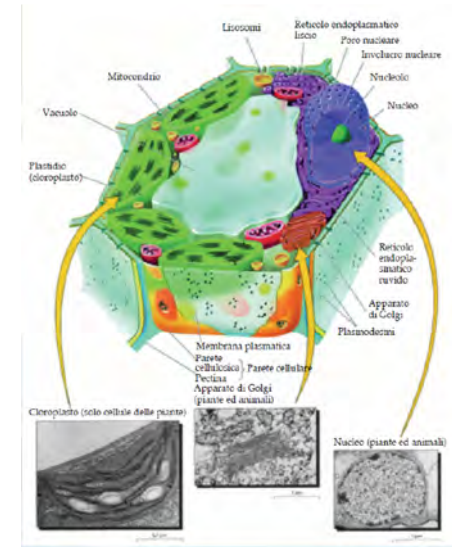


La FCM applicata al mondo vegetale (1973)

Primo articolo sull'applicazione della citometria a flusso all'analisi di cellule vegetali fu pubblicato nel 1973, ma solo a partire dagli anni 80 si diffonde maggiormente l'uso del citometro a flusso in ambito vegetale

Criticità iniziali:

- Difficoltà nella preparazione di cellule in sospensione da tessuti vegetali costituiti da cellule tenute insieme da una matrice extracellulare
- Presenza della parete cellulare che conferisce forma non sferica alle cellule: flusso laminare disturbato, traiettoria cellule e interazione con raggio luminoso variabile.
- Bassa permeabilità della parete cellulare ai reagenti.
- Elevate dimensioni cellulari, a volte superiori all' orifizio del nozzle
- Presenza di un'ampia gamma di metaboliti secondari che possono interferire con la colorazione della cellula e dei suoi componenti (polifenoli, pigmenti)

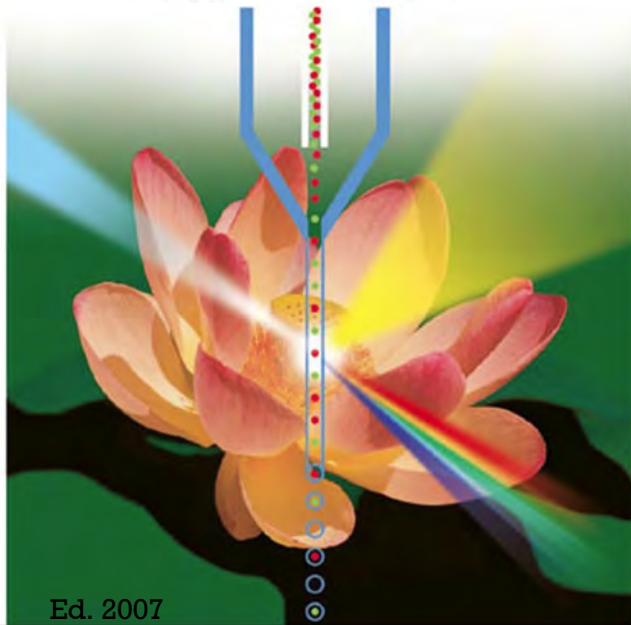


Ambiti di applicazione

Edited by Jaroslav Doležel,
Johann Greilhuber and Jan Suda

WILEY-VCH

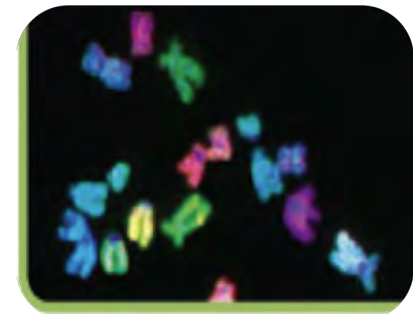
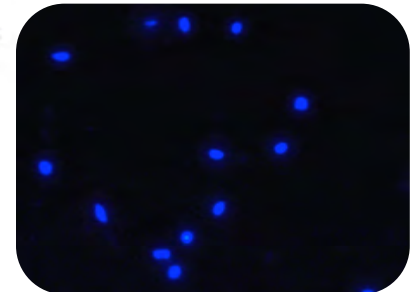
Flow Cytometry with Plant Cells



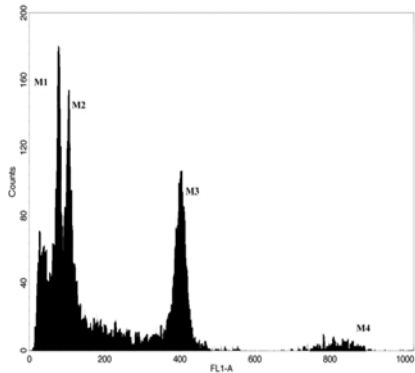
Ed. 2007

ENEA

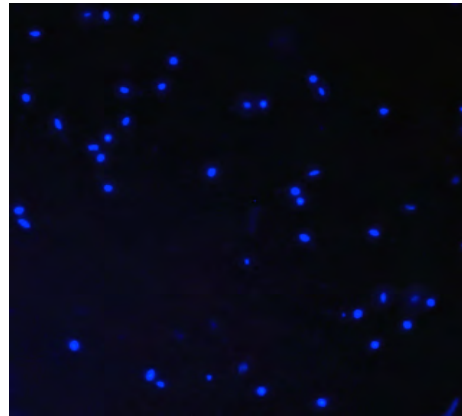
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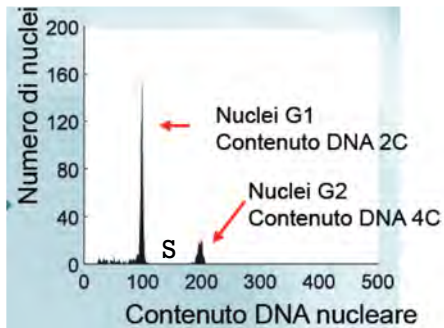
Esempi di applicazioni CF in ambito vegetale



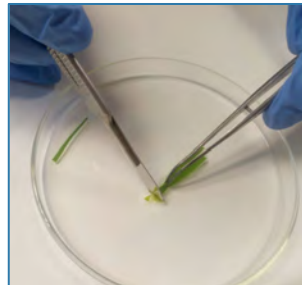
Determinazione contenuto di DNA



← **Nuclei** →

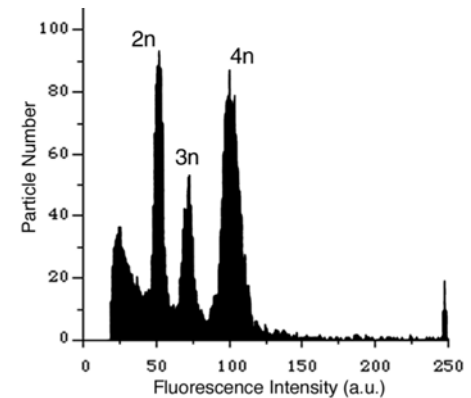
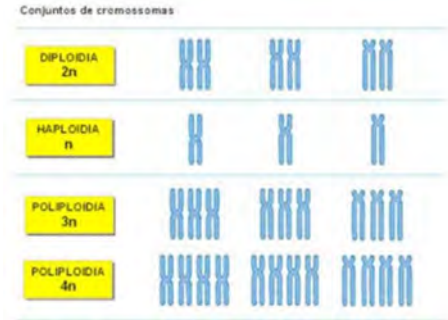


Analisi ciclo cellulare



(Galbraith et al., 1983)

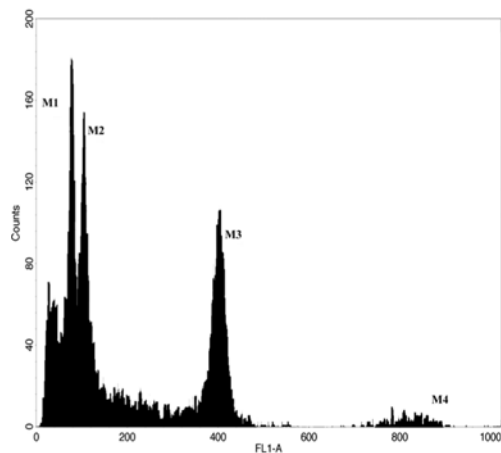
Analisi livello di ploidia



Determinazione contenuto di DNA in Cardo e Carciofo (*Cynara cardunculus* var.)

One Major Challenge of Sequencing Large Plant Genomes Is to Know How Big They Really Are

(Dolezel et al. 2018)



Determinazione contenuto di DNA in nuclei di cardo (M1) e carciofo (M2) colorati con ioduro di propidio. M3 standard di riferimento (*Pisum sativum*)
(da Giorgi et al. 2016)

Standard di riferimento

Plant Species and Cultivar *	2C DNA Content (pg DNA) **
<i>Raphanus sativus</i> L. 'Saxa'	1.11
<i>Solanum lycopersicum</i> L. 'Stupické polní rané'	1.96
<i>Glycine max</i> Merr. 'Polanka'	2.50
<i>Zea mays</i> L. 'CE-777'	5.43
<i>Pisum sativum</i> L. 'Ctirad'	9.09
<i>Secale cereale</i> L. 'Daňkovské'	16.19
<i>Vicia faba</i> L. 'Inovec'	26.90
<i>Allium cepa</i> L. 'Alice'	34.89

(da Dolezel et al. 2018)

$$\text{Contenuto 2C del DNA del campione} = \left[\frac{\text{media del picco } G_1 \text{ del campione}}{\text{media del picco } G_1 \text{ dello standard}} \right] \times \text{contenuto 2C del DNA dello standard (pg DNA)}$$

RISULTATI

Contenuto 2C DNA cardo = $2.20 \text{ pg} \pm 0.04$

Contenuto 2C DNA carciofo = $2.40 \text{ pg} \pm 0.04$

Analisi livello di ploidia

L'esempio dei grani: *Triticum sp*



T. urartu

Genoma (AA)
($2n=2x=14$)



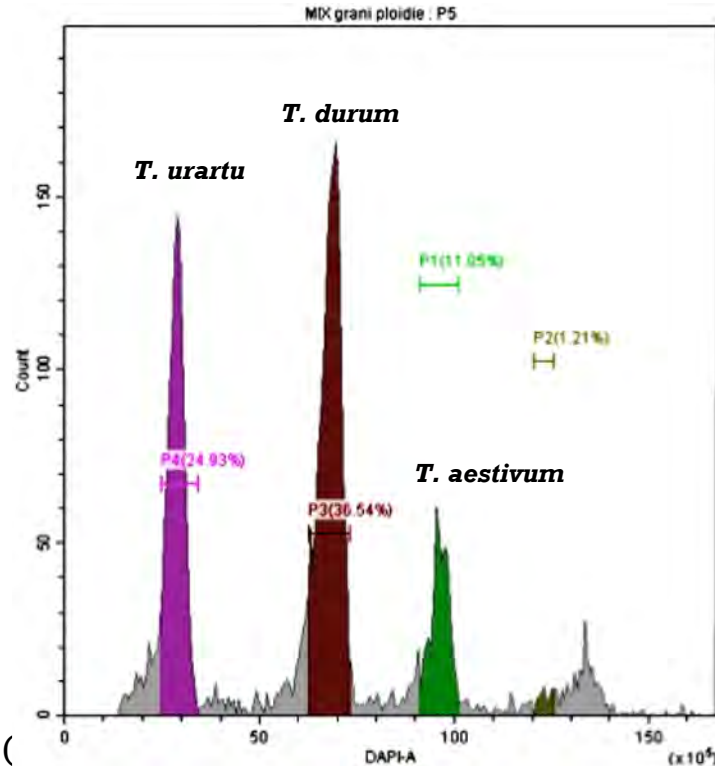
T. durum

Genoma AABB)
($2n=4x=28$)



T. aestivum

Genoma (AABBDD)
($2n=6x=42$)



Conjuntos de cromosomas



FCM e Plant Breeding

Induzione artificiale di poliploidi

- Maggiore vigore rispetto ai parentali
- Incremento nella dimensioni di alcuni organi e nella produzione di alcuni metaboliti
- Migliore capacità di adattamento



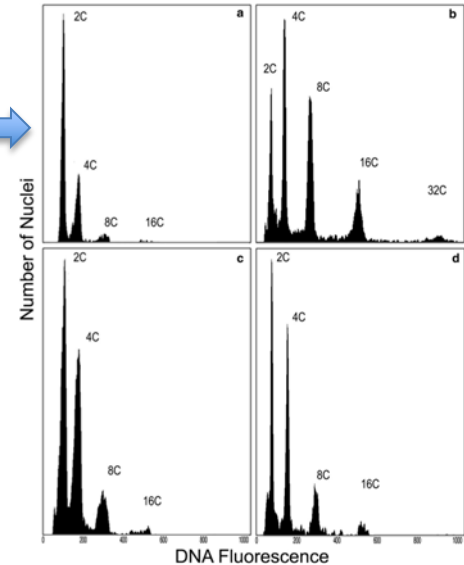
Protocorm like bodies (PLBs) in sospensione



Totipotenza delle cellule vegetali

Table 1 PLBs polyloidization and FCM characterization of ploidy levels on in vitro explants

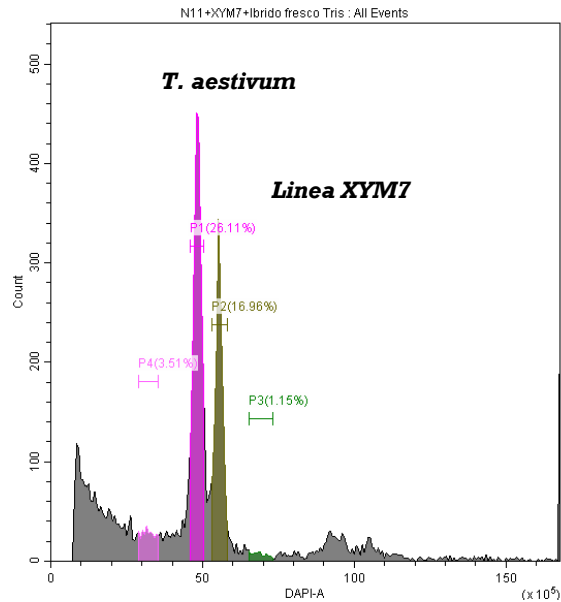
Antimissic/concentration	Duration time (d)	PLBs and plantlets explants			FCM analysis and polyploids recovery			
		Number of initial explants	Number of final explants ¹	Number of regenerated plantlets ²	Cycle Value ³	Recovery of poly-ploid plantlets (%) ⁴	TT plants (%) ⁵	PEP plants (%) ⁶
Control	–	30	340	78	0.5 ± 0.15 ¹	0	0	0
COL 0.025%	3	30	400	82	1.51 ± 0.22 ²	60	50	50
	10	30	390	60	0.76 ± 0.12 ²	14	100	0
	21	30	0	0	–	–	–	–
COL 0.050%	3	30	310	61	1.47 ± 0.11 ¹	15	85	15
	10	30	305	33	1.01 ± 0.08 ²	0	0	0
	21	30	0	0	–	–	–	–
COL 0.075%	3	30	390	92	1.59 ± 0.17 ²	80	40	60
	10	30	210	56	0.96 ± 0.09 ²	20	20	0
	21	30	0	0	–	–	–	–
APM 1.25 µM	3	30	290	92	0.52 ± 0.08 ¹	0	0	0
	10	30	150	65	0.86 ± 0.12 ²	0	0	0
	21	30	0	0	–	–	–	–
APM 2.5 µM	3	30	590	155	1.1 ± 0.14 ¹	55	100	0
	10	30	145	79	0.98 ± 0.09 ²	0	0	0
	21	30	0	0	–	–	–	–



A high-throughput flow cytometry system for early screening of in vitro made polyploids in *Dendrobium* hybrids

FCM e “Plant Breeding”

Analisi incroci interspecifici



Triticum aestivum
AABBDD



Secale cereale
(RR)

X



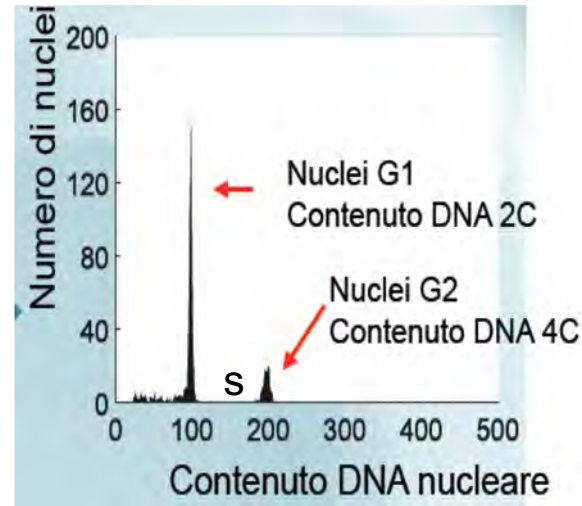
Analisi GISH; in rosso cromosomi di segale in blu di frumento

Linea XYM7 AABB(RR)2D2D

Analisi ciclo cellulare

Per:

- Studiare effetti di composti citotossici (allelopatia, blocco sviluppo spore fungine...)
- Studiare stadio fisiologico del seme: sviluppo, maturazione, germinazione
- Monitorare priming/conservazione



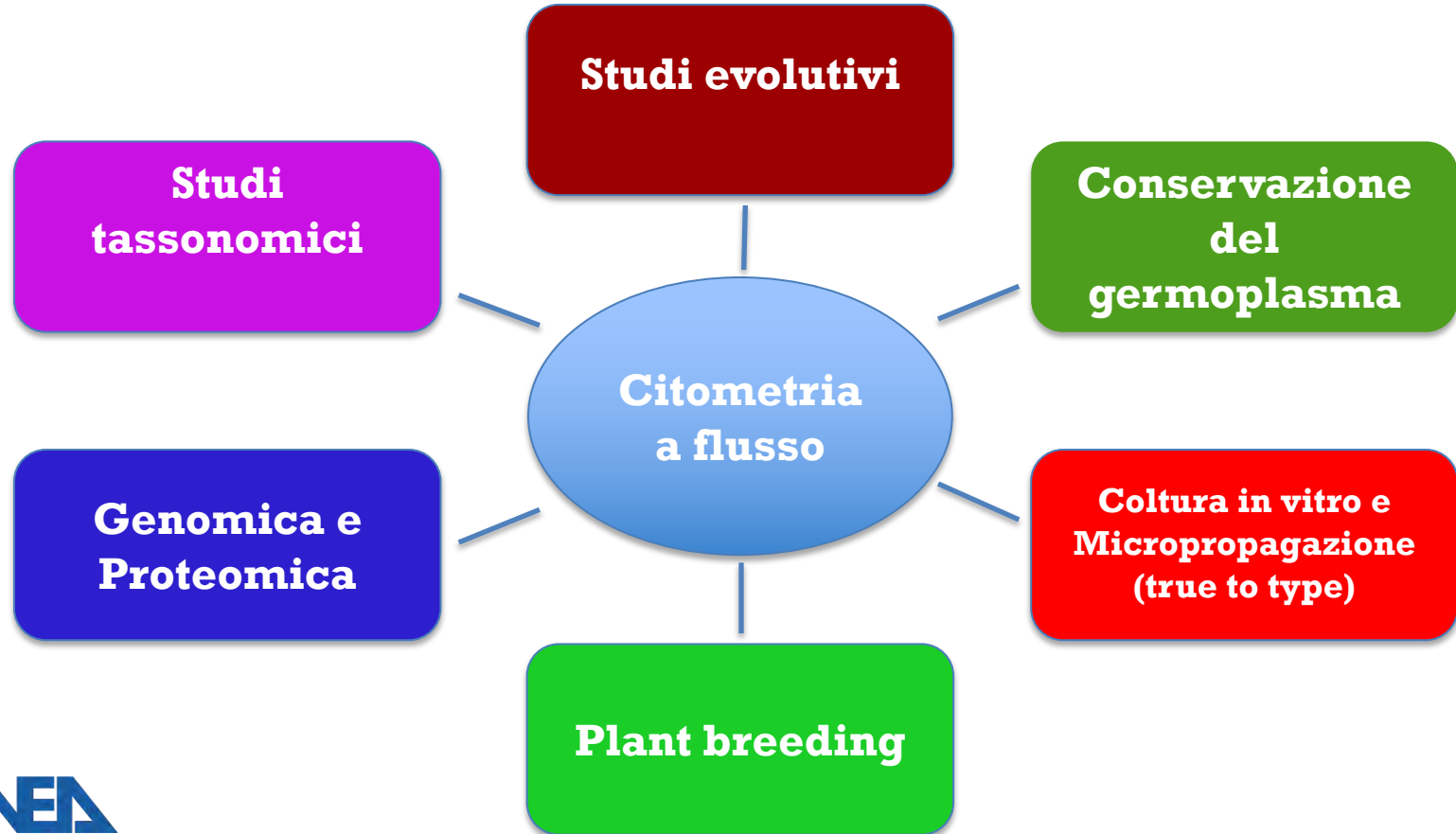
$$(4C/2C) = \frac{\text{n}^\circ \text{ nuclei con 4C DNA}}{\text{n}^\circ \text{ nuclei con 2C DNA}}$$

REVIEW

Flow cytometry – a modern method for exploring genome size and nuclear DNA synthesis in horticultural and medicinal plant species

Elwira Sliwinska 2018. DOI: 10.2478/fhort-2018-0011

FCM a supporto di...



Cromosomi mitotici e Citogenetica a flusso

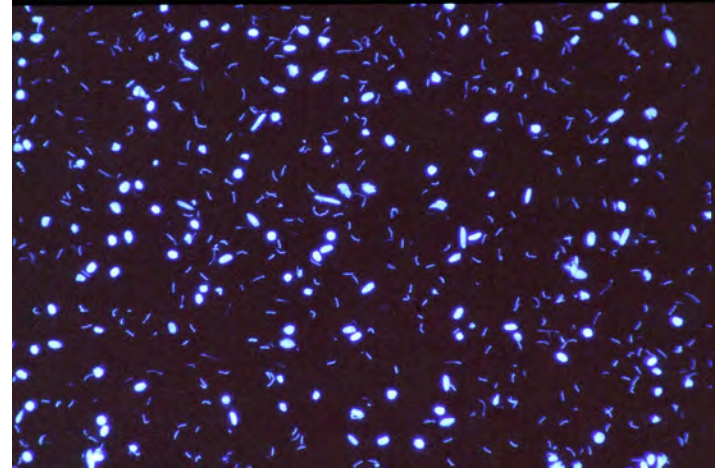
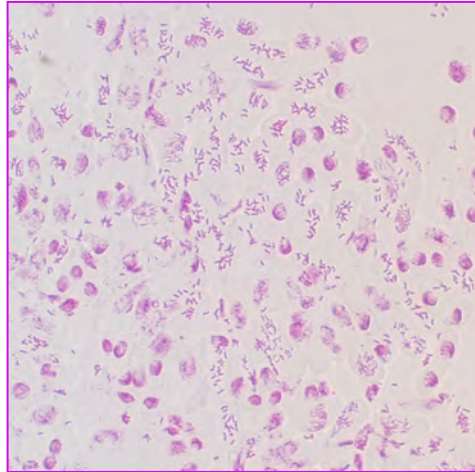
A high-yield procedure for isolation of metaphase chromosomes from root tips of *Vicia faba* L.

J. Doležel¹, J. Čihalíková¹, and S. Lucretti²

¹ Institute of Experimental Botany, Department of Plant Biotechnology, Sokolovská 6, CS-77200 Olomouc, Czechoslovakia

² ENEA, C.R.E. Casaccia, Genetic Engineering Division, C.P. 2400, I-00100 Rome, Italy

• *Planta* (1992) 188:93–98

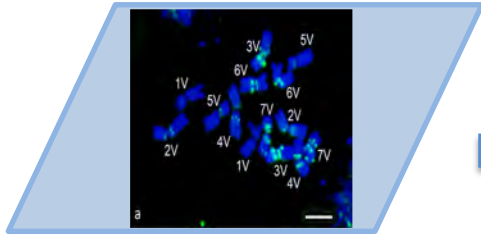


Colorazione Feulgen apici radicali di grano Sospensione di cromosomi e nuclei di grano.

DNA "colorato" con DAPI

Citogenetica a flusso

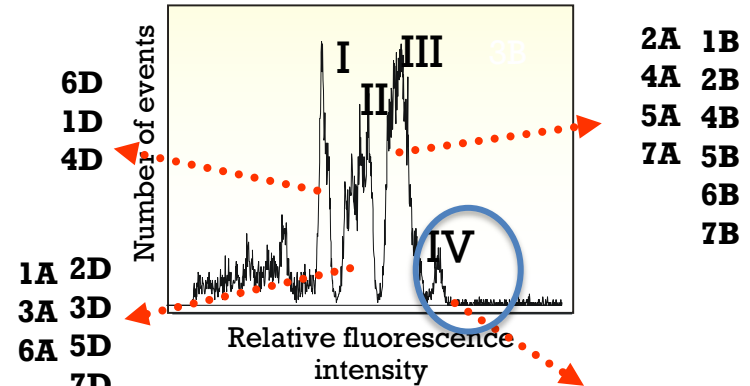
Dal vetrino ...



Cariotipo ideale



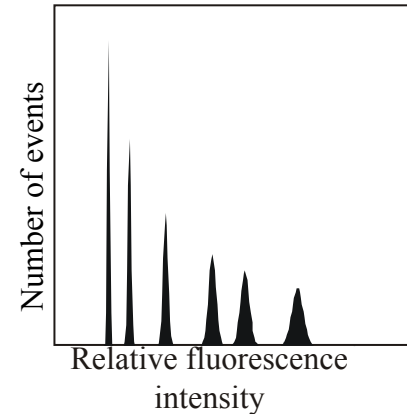
Cariotipo a Flusso



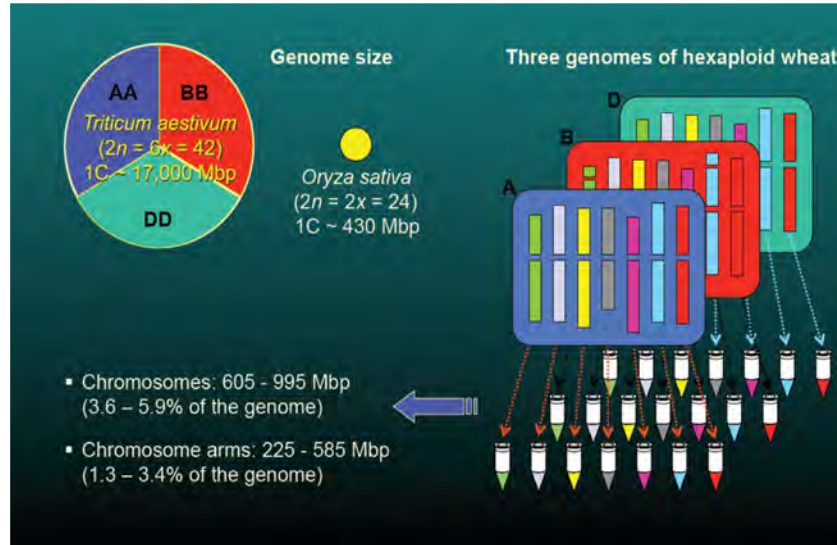
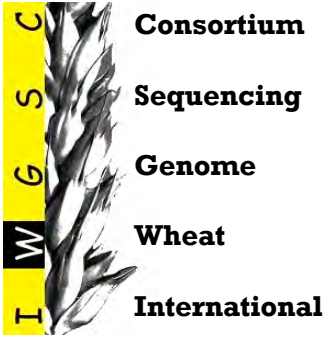
Cariotipo a flusso ideale

2A 1B
4A 2B
5A 4B
7A 5B
6B
7B

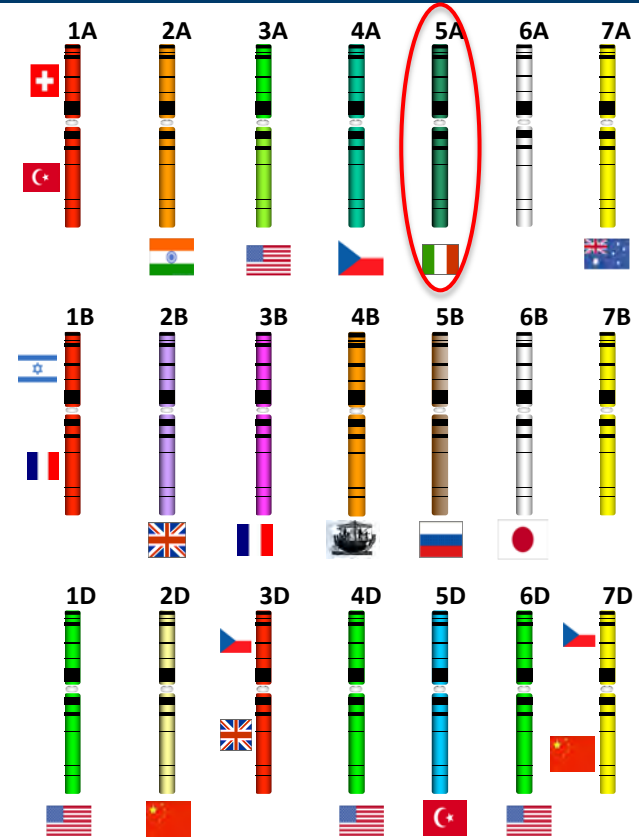
3B



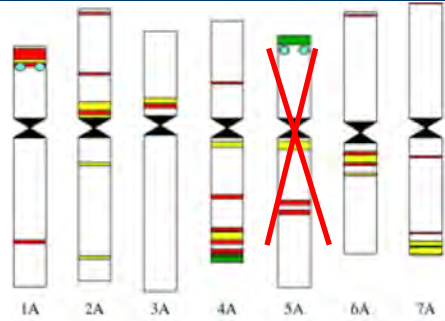
L'approccio cromosomico per il sequenziamento del grano tenero



Doležel 2009 presentazione orale

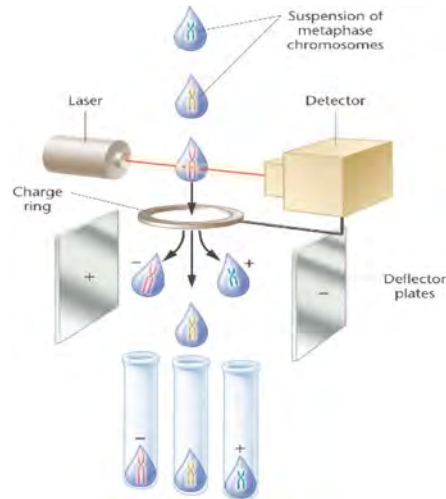


Linee aneuploidi in grano e l'approccio cromosomico

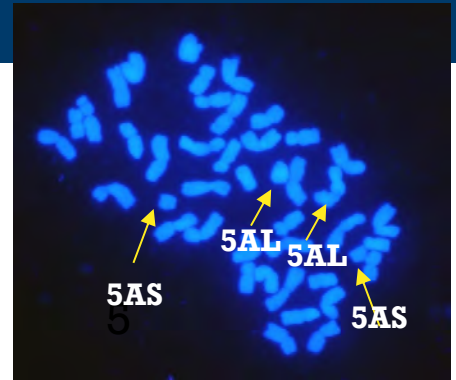


**Uso di Linee aneuploidi
Linee ditelosomiche**

Analisi CSDt5A

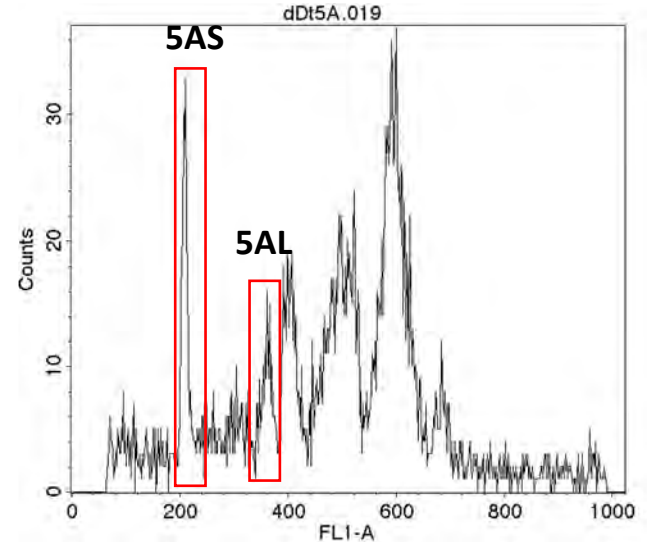
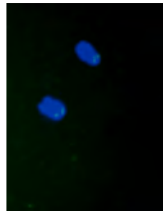
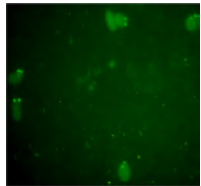
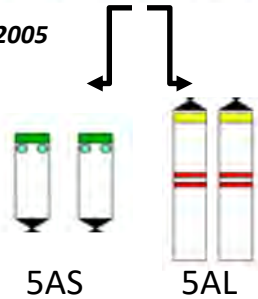


sorting



CSDt5A metafase

Da Kubalàková et al. 2005



Principali e potenziali utilizzazioni dei cromosomi “sortati”

Biotechnology Advances 32 (2014) 122–136

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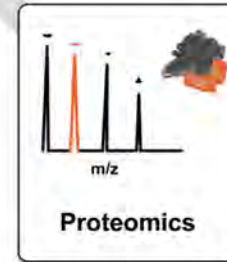
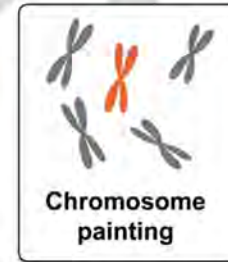
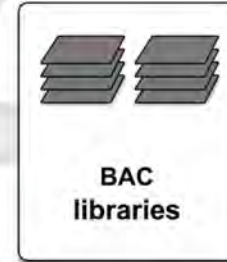
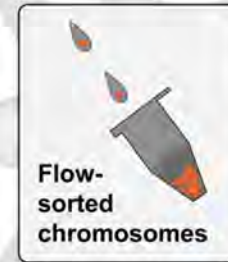
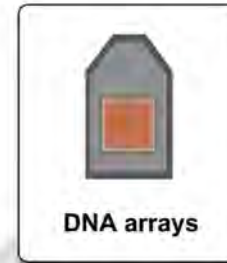
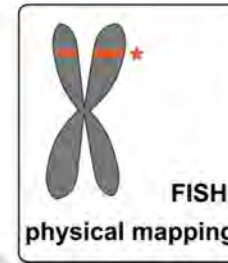
Research review paper

Advances in plant chromosome genomics

Jaroslav Doležel*, Jan Vrána, Petr Čápal, Marie Kubaláková, Veronika Burešová, Hana Šimková

Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany, Šlechtůvův 31, Olomouc CZ-78771, Czech Republic

CrossMark



L'approccio cromosomico: problematiche

- Non per tutte le specie sono disponibili linee aneuploidi
- Le linee aneuploidi sono spesso ottenute in varietà modello
- e non in varietà di interesse agronomico

Quale soluzione?



OPEN ACCESS Freely available online

PLOS ONE

FISHIS: Fluorescence *In Situ* Hybridization in Suspension and Chromosome Flow Sorting Made Easy

Debora Giorgi¹, Anna Farina¹, Valentina Grosso¹, Andrea Gennaro², Carla Ceoloni², Sergio Lucretti^{1*}

¹ ENEA – Italian National Agency for New Technologies, Energy and Sustainable Economic Development, CASACCIA Research Center, Rome, Italy, ² DAFNE – Department of Agriculture, Forestry, Nature and Energy, University of Tuscia, Viterbo, Italy (2014)

Confronto FISH - FISHIS

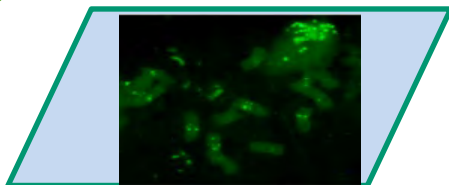
FISH : standard procedure

Probe **X** labelling

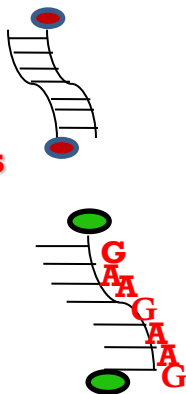
Single strand oligonucleotides synthetically labeled derived from microsatellites



Microscope observation

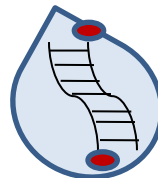


ENEA



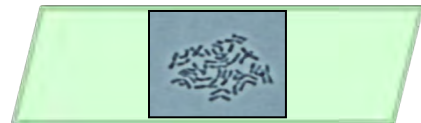
Hybridization mix

50% Formamide
salmon sperm DNA (blocking)
10% dextrano solphate
2XSSC
probe []



FISHIS

spread metaphase



slide

DNA **X** denaturation at 75-80C

Alkaline DNA denaturation at pH 13* for 20 min followed by Tris.HCl neutralization

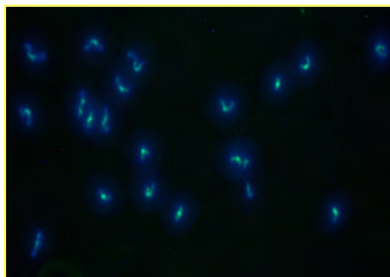
Detection Staining

Washing **X** steps

Hybridization **X** o.n. at 37°C

Hybridization at r.t. for 1 h

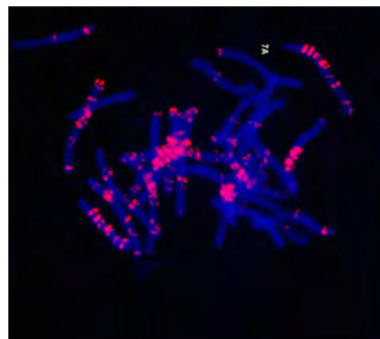
Da FISH a FISHIS: nascita della Citogenetica molecolare a flusso



$(GAA)_7$ FISHIS

Possibilità di estendere l'approccio cromosomico a specie selvatiche e varietà di interesse agronomico

L'esempio del grano duro

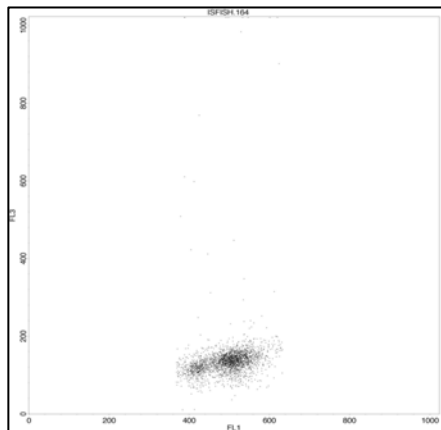


Ibridazione FISH su metafase di *Triticum durum* ($2n=4x=28$ AABB) sonda $(GAA)_7$ Cy3



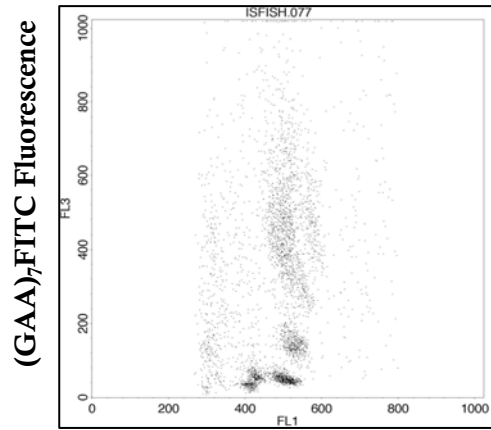
FSC

Standard dot plot



Fluorescenza DNA (DAPI)

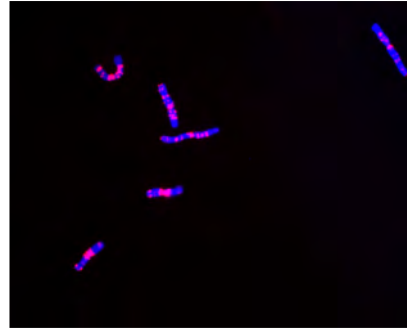
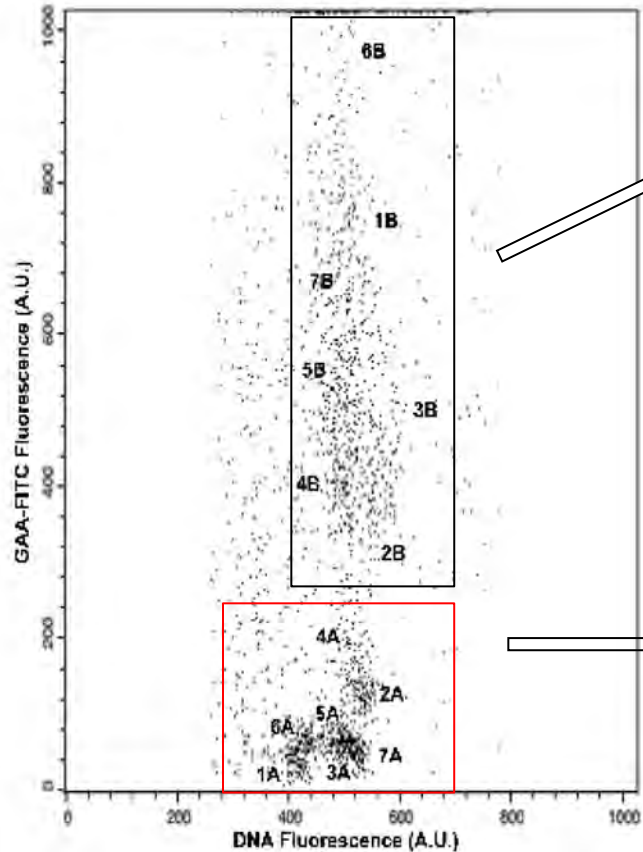
Dot plot dopo FISHIS



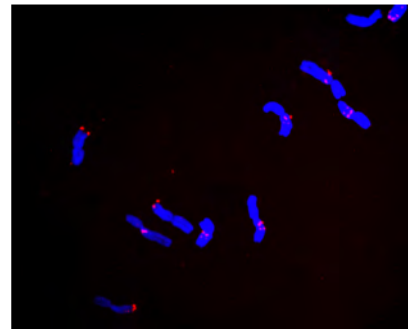
Fluorescenza DNA (DAPI)



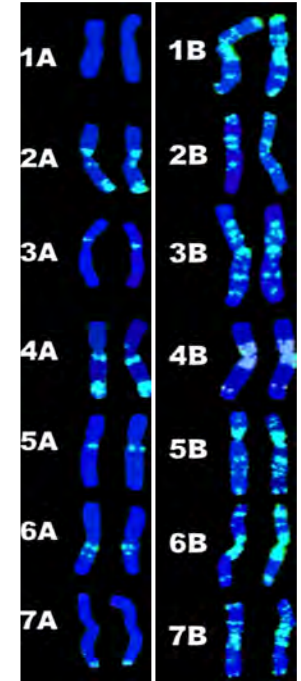
FISHIS e separazione dei genomi A e B in grano duro



Genome B



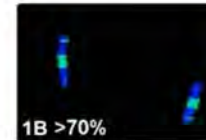
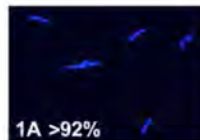
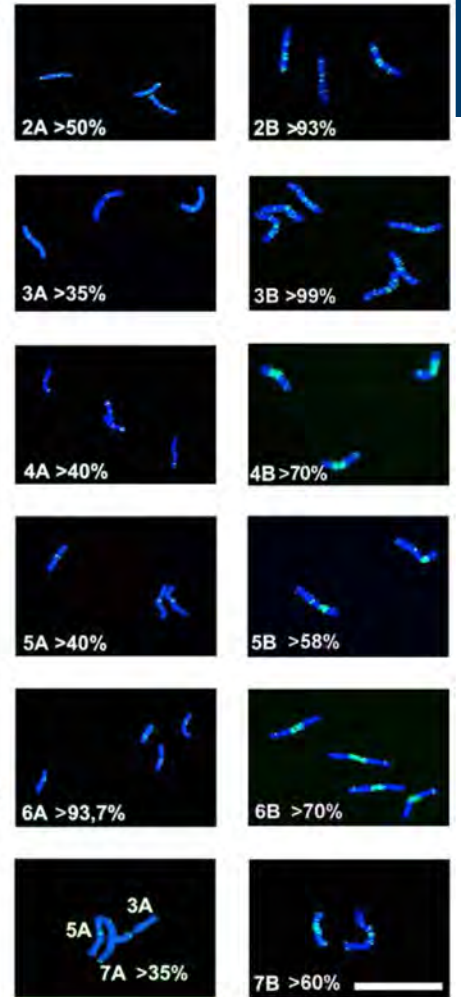
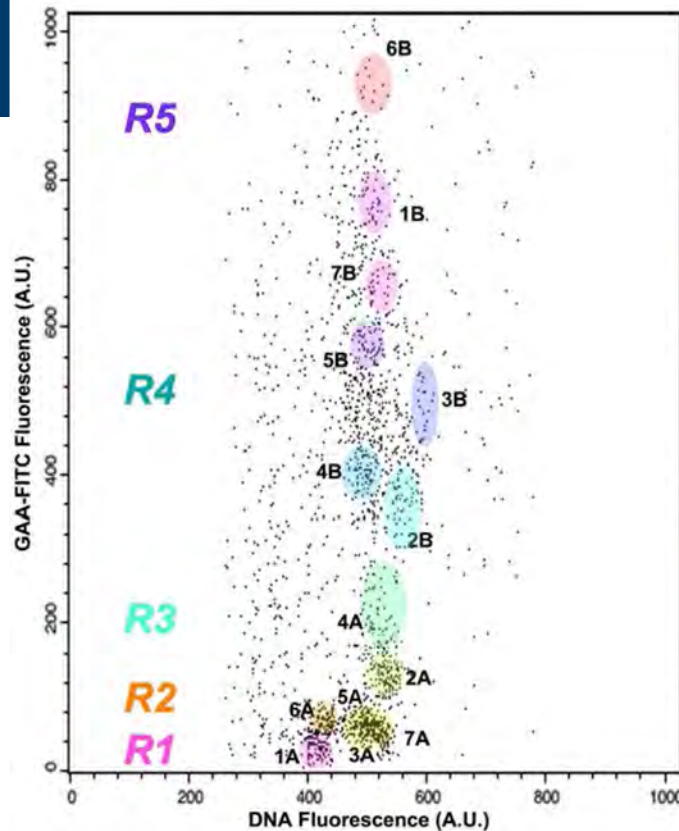
Genome A



(GAA)₇ distribution
on
pasta wheat
genomes

Chromosome sorting

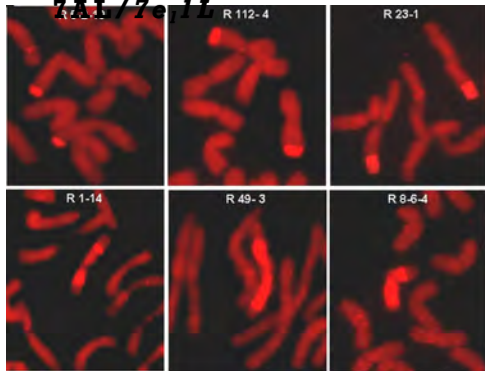
Separazione di singoli cromosomi appartenenti ai due genomi A e B di grano duro



Sorting di cromosomi “alieni” da linee ricombinanti

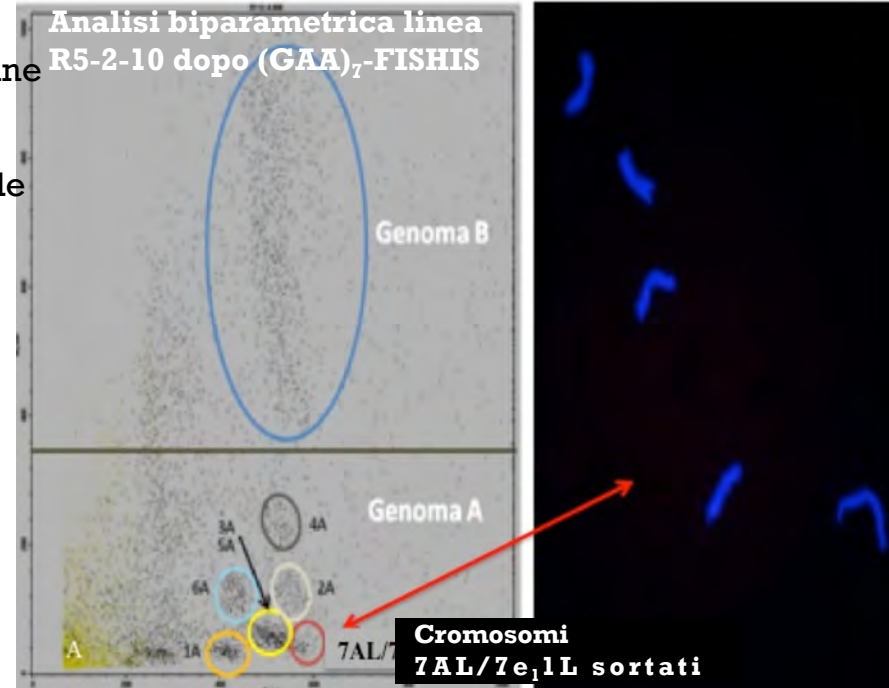
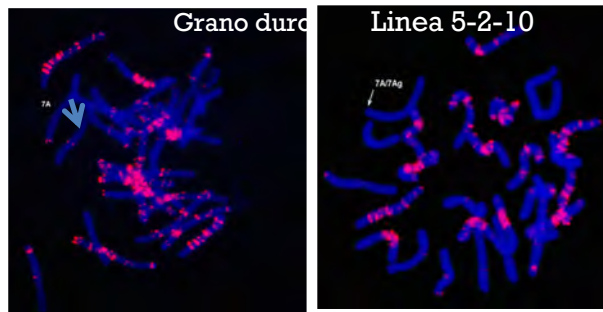
Linee ricombinanti *Triticum durum*/*Thinopyrum ponticum* ottenute mediante ingegneria cromosomica (Ceoloni et al. 2014)

Linee ricombinanti

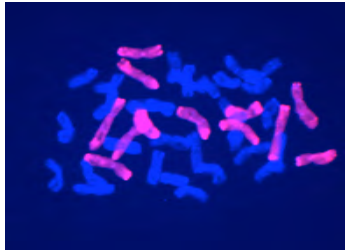


Ceoloni et al. 2014

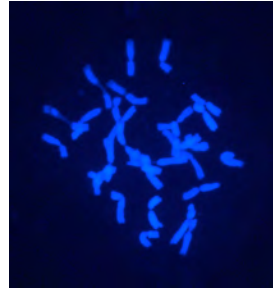
- Geni resistenza ruggine
- QTL per produttività
- Colorazione cariosside



FISHIS e FCM per l'analisi di “cromosomi alieni”



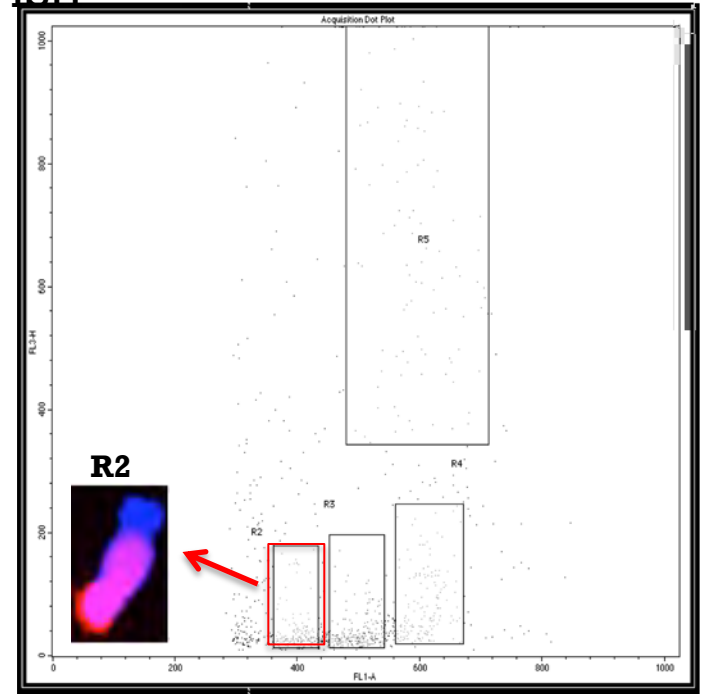
X n reincroci
con *T. aestivum*



T. aestivum

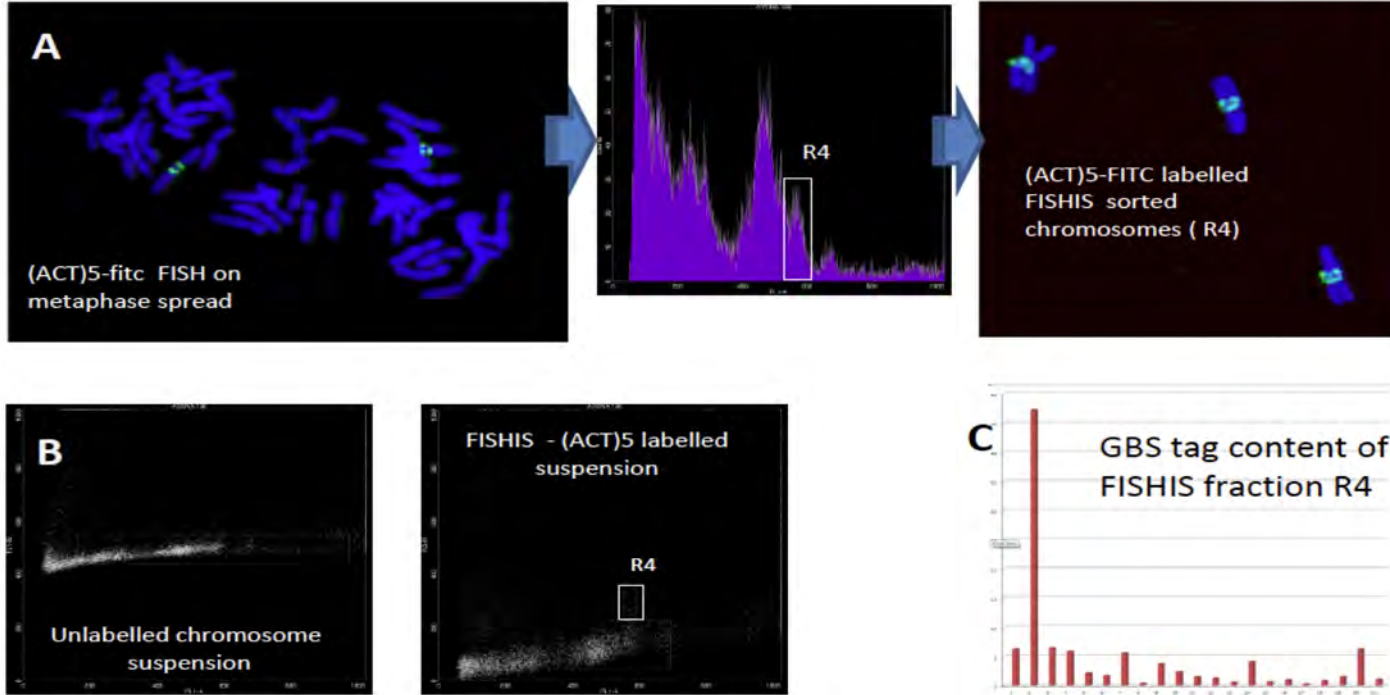


Sorting cromosomico dopo $(GAA)_7$
FISH



Citogenetica molecolare a Flusso e Genotyping by sequencing (GBS)

In *Avena sativa*



FCM in pianta: passato, presente e ...futuro

REVIEW ARTICLE

Cytometry

Flow Cytometry in Plant Breeding

Sergio J. Ochatt*
2008

the plant journal



The Plant Journal (2019)

doi: 10.1111/tpj.14286

TECHNICAL ADVANCE

Direct analysis of pollen fitness by flow cytometry: implications for pollen response to stress

Glad Luria^{1,2}, Nicholas Rutley^{1,2}, Itay Laza¹, Jeffery F. Harper² and Gad Miller^{1,2}

¹The Mina and Everard Goodman, Faculty of Life Sciences, Bar Ilan University, Ramat Gan 5290002, Israel, and

²Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV, USA

Planta (2018) 248:279–291

<https://doi.org/10.1007/s00425-018-2901-2>

ORIGINAL ARTICLE



Application of flow cytometry with a fluorescent dye to measurement of intracellular nitric oxide in plant cells

Jan Kępczyński¹ · Danuta Cembrowska-Lech¹

Chapter from book *Applied Plant Cell Biology: Cellular Tools and Approaches for Plant Biotechnology* (pp.395-430)

Flow Cytometry in Plant Research: A Success Story

Chapter · January 2014 with 254 Reads

DOI: [10.1007/978-3-642-41787-0_13](https://doi.org/10.1007/978-3-642-41787-0_13)

Publisher: 1861-1370

J Plant Res (2018) 131:179–189

DOI: [10.1007/s10265-017-0975-8](https://doi.org/10.1007/s10265-017-0975-8)



REGULAR PAPER

Relaxed chromatin induced by histone deacetylase inhibitors improves the oligonucleotide-directed gene editing in plant cells

Hilda Tiricz¹ · Bettina Nagy¹ · Györgyi Ferenc¹ · Katalin Török¹ · István Nagy^{2,3} · Dénes Dudits¹ · Ferhan Ayaydin^{1,4}

Plant, Cell & Environment



ORIGINAL ARTICLE

Cell cycle acceleration and changes in essential nuclear functions induced by simulated microgravity in a synchronized *Arabidopsis* cell culture

Khaled Y. Kamal, Raül Herranz, Jack J.W.A. van Loon, F. Javier Medina

First published: 14 August 2018 | <https://doi.org/10.1111/pce.13422>



continua...



Sergio Lucretti



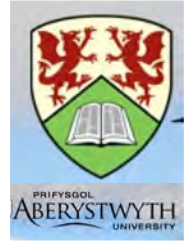
Paola Crinò

Grazie per l'attenzione



**Consortium
Sequencing
Genome
Wheat
International**

Tim Langdon



Valentina Grosso



Anna Farina

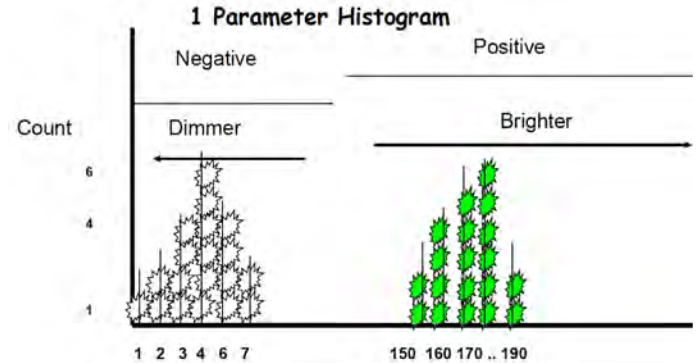
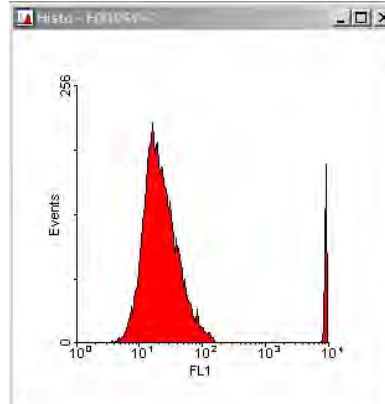
sergio.lucretti@enea.it
debora.giorgi@enea.it
anna.farina@enea.it



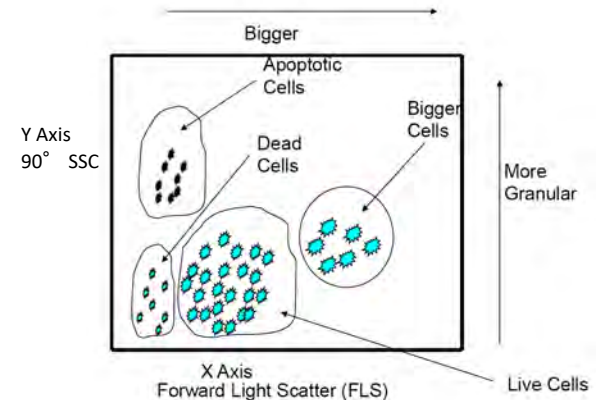
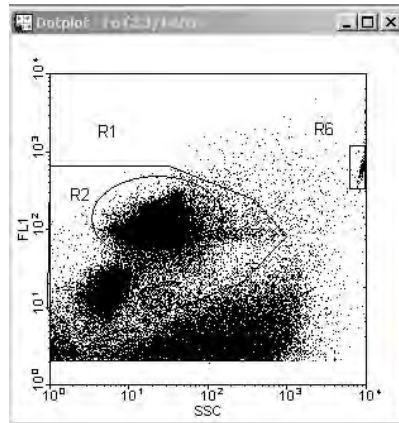
**Carla Ceoloni
Liliana Kusmanovic
Domenico Lafiandra
Francesco Sestili**

Rappresentazione dei dati

Istogramma (analisi singolo parametro)



Dot Plot analisi bi-parametrica

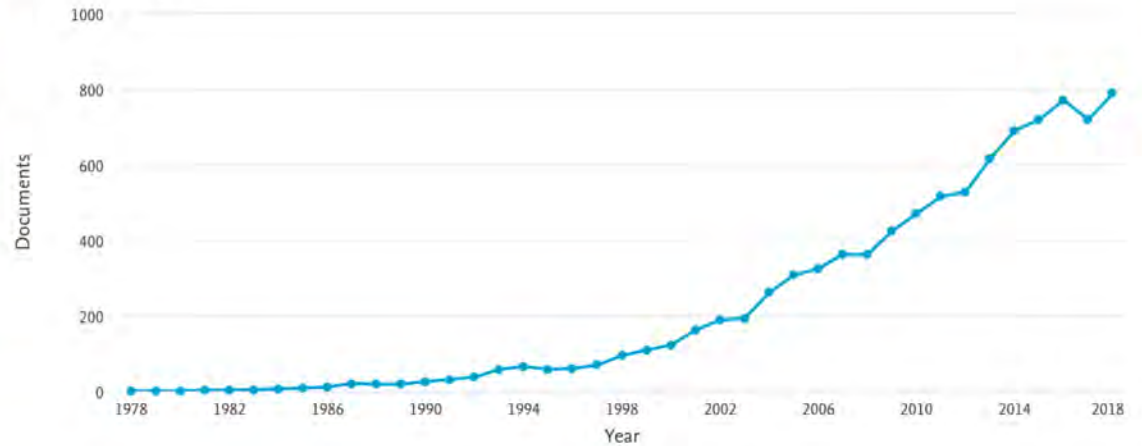


9,212 document results

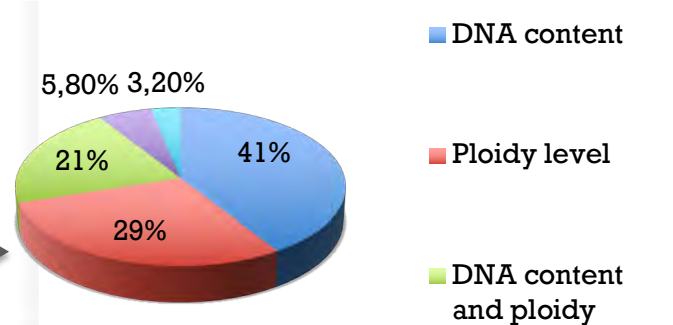
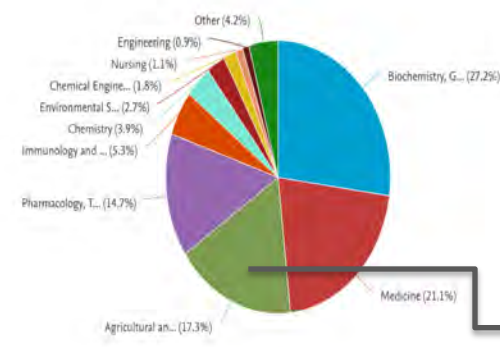
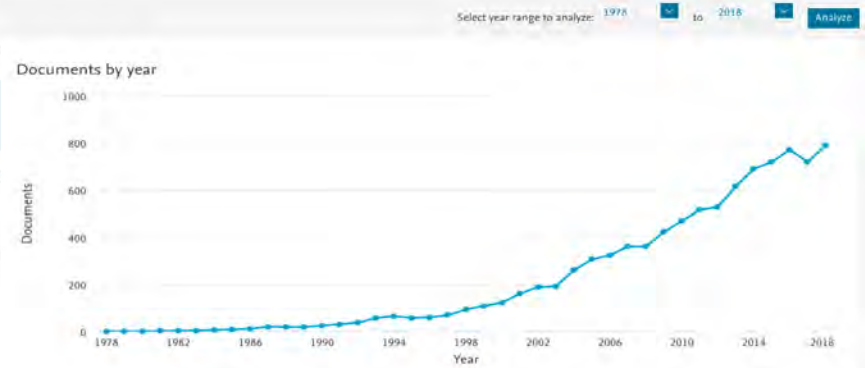
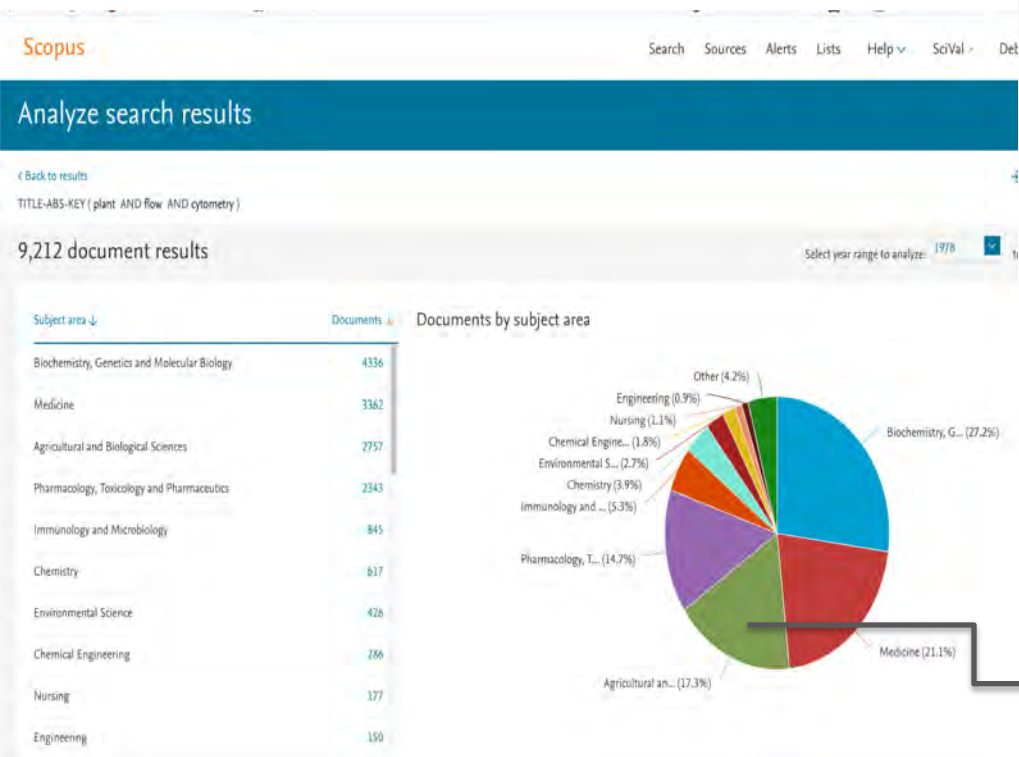
Select year range to analyze: 1978 to 2018 [Analyze](#)

Year	Documents
2018	788
2017	718
2016	771
2015	718
2014	690
2013	615
2012	527
2011	515
2010	470
2009	423

Documents by year



Plant flow cytometry in letteratura



Flow cytometry in plant science



Cell nuclei

Ploidy, genome size, cell cycle kinetics, apoptosis, endopolyploidy, gene expression, HNW DNA isolation



Mitotic chromosomes

Chromosome sorting for genome mapping and sequencing



Protoplasts

Sorting of heterokaryons, analysis of gene expression, uptake of plasmids and viruses



Pollen grains

Optical properties of pollen, sorting embryogenic male sperm cells, pollen viability



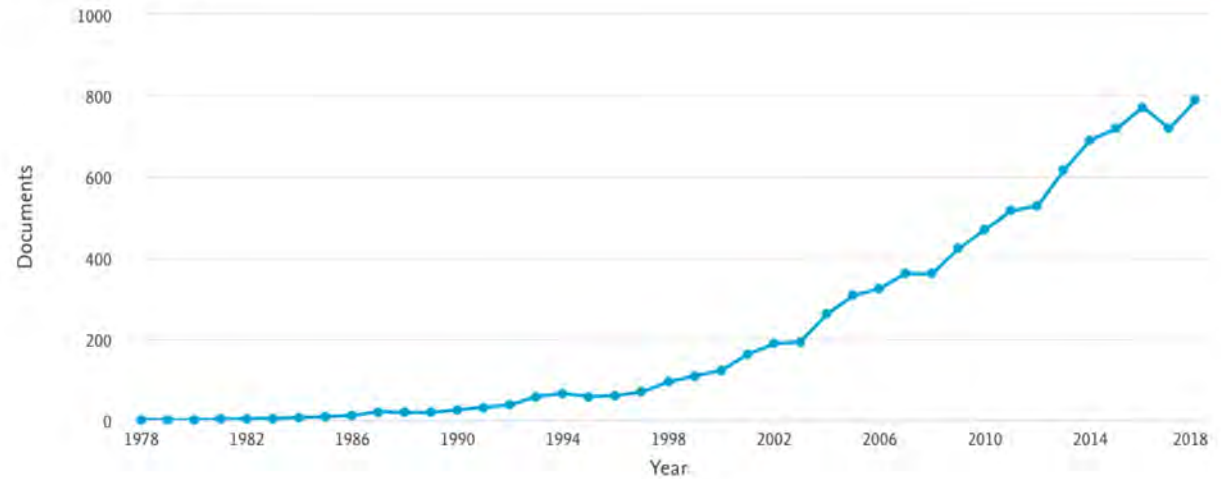
Chloroplasts and mitochondria

Optical properties of chloroplasts, membrane potential of mitochondria

Da Scopus: Plant Flow cytometry

Select year range to analyze: 1978 to 2018 Analyze

Documents by year



Although the first patent on a prototype was issued in 1953, the first flow cytometry device based on fluorescence was developed in 1968 by Wolfgang Göhde from the University of Muenster (Germany) and was first commercialized in 1968–69 by Partec GmbH (Muenster, Germany) through PHYWE AG (Goettingen, Germany).

Although the first paper on plant nuclei analysis using flow cytometry was published in 1973 the breakthrough in plant DNA analysis by FC came during the late 1980s, when researchers started to persistently apply this technology in plants.

Flow cytometry is an excellent method for detailed analysis of proliferating cell populations as it provides the distribution of nuclei based on their DNA content (Youngh et al 2016)

Fluorescent stains for DNA

Fluorescent Dye	Primary Binding Mode	Wavelength (nm)*	
		Excitation	Emission
Ethidium bromide**	Intercalation	530	605
Propidium iodide**	Intercalation	540	615
Hoechst 33258	AT-binding	365	465
Hoechst 33342	AT-binding	360	460
DAPI	AT-binding	365	450
DIPI	AT-binding	365	450
Chromomycin A3	GC-binding	445	570
Mihtramycin	GC-binding	445	575
Olivomycin	GC-binding	440	560