TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

Dr. Prabhakar Singh
Department of Biochemistry
Faculty of Science, V.B.S. Purvanchal University, Jaunpur
PIN-222 003, (U.P.), INDIA.
Mobile No.: +91-9454695363
E-Mail: pruebiochem@gmail.com

VEER BAHADUR SINGH PURVANCHAL UNIVERSITY JAUNPUR-222003

E –content

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Prepared by: Dr. Prabhakar Singh
Department : Biochemistry
Faculty : Science
Email: pruebiochem@gmail.com
Contact: +91-9454695363

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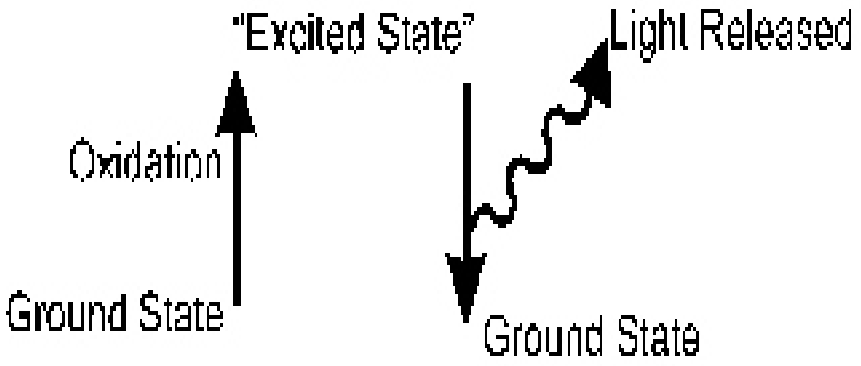
FLUORESCENCE

Fluorescence is an emission phenomenon where an energy transition from a higher to a lower state is accompanied by radiation. Only molecules in their excited forms are able to emit fluorescence; thus, they have to be brought into a state of higher energy prior to the emission phenomenon.

The fluorescent group in a molecule is called a **fluorophore.**

The ratio of photons emitted and photons absorbed by a fluorophore is called **quantum yield**



Principle

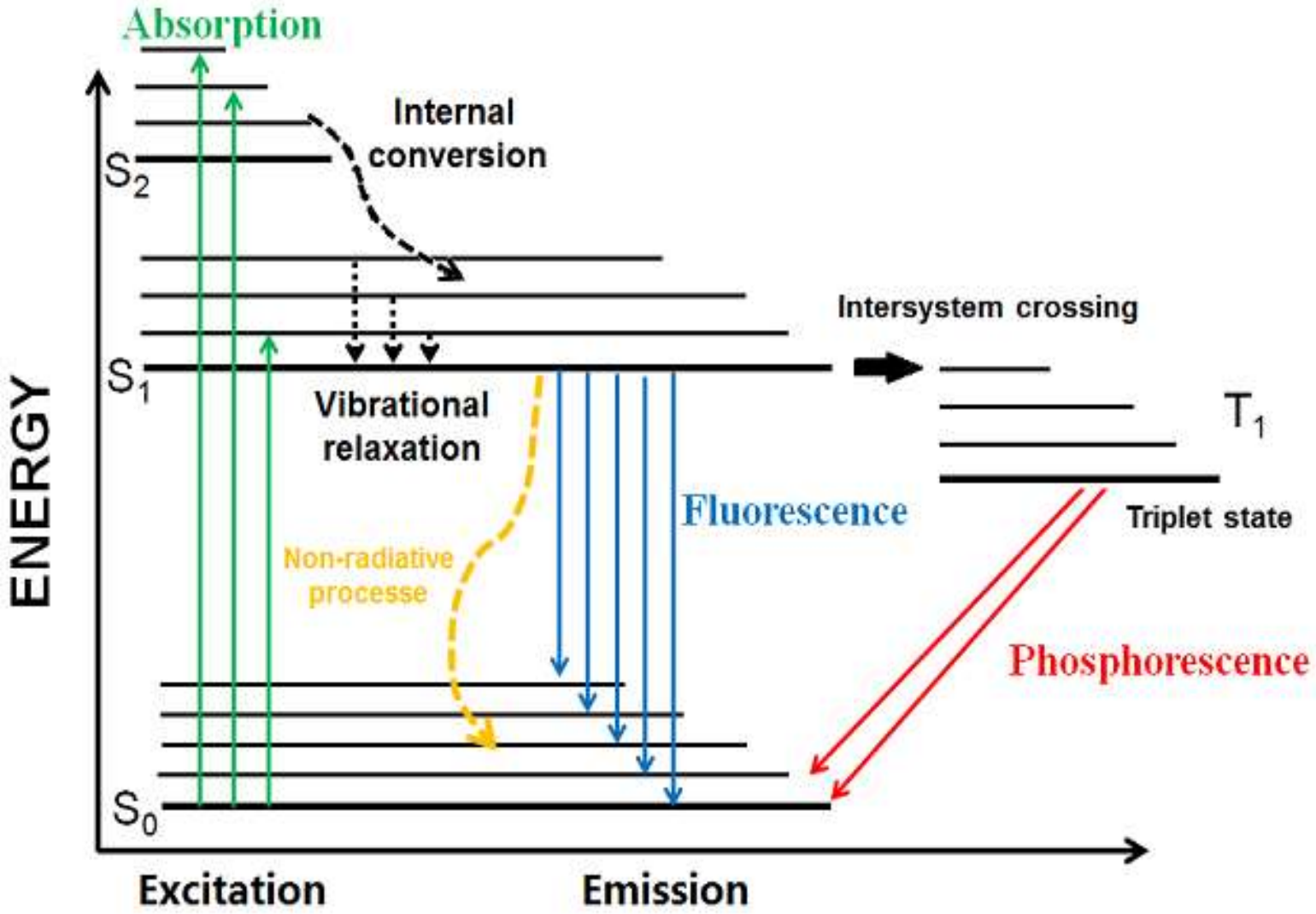
- A molecule in its electronic and vibrational ground state (S0v0) can absorb photons matching the energy difference of its various discrete states.The excess energy is absorbed as vibrational energy (v>0), and quickly dissipated as heat by collision with solvent molecules. The molecule thus returns to the vibrational ground state (S1v0).

- These relaxation processes are non-radiating transitions from one energetic state to another with lower energy, and are called internal conversion (IC). From the lowest level of the first electronic excited state, the molecule returns to the ground state (S0) either by emitting light (fluorescence) or by a non-radiative transition.

- Radiative energy is lost in fluorescence as compared to the absorption, hence, the fluorescent light is always at a longer wavelength than the exciting light (Stokes shift).

- **Phosphorescence :** An associated phenomenon in this context is phosphorescence which arises when the molecule gets into the triplet state from an electronic excited singlet state by a process called intersystem crossing (ISC).

  The rate constants for phosphorescence are much longer and phosphorescence thus happens with a long delay and persists even when the exciting energy is no longer applied



Jablonski diagram. Shown are the electronic ground state (S0), two excited singlet states (S1,S2) and a triplet state (T1). Vibrational levels (v) are only illustrated exemplarily. Solid vertical lines indicate radiative transitions, dotted lines show non-radiative transitions.

LUMINESCENCE

- The term 'luminescence' was introduced in 1888 by Eilhard Wiedemann. Historically, radioactivity was thought of as a form of "radio-luminescence", although it is today considered to be separate since it involves more than electromagnetic radiation.

- **Luminescence** is emission of light by a substance not resulting from heat; it is thus a form of cold body radiation.

- It can be caused by chemical reactions, electrical energy, subatomic motions, or stress on a crystal.

- This distinguishes luminescence from incandescence, which is light emitted by a substance as a result of heating.

Chemiluminescence: A result of a chemical reaction

Bioluminescence: A result of biochemical reaction by a living organism

Electrochemiluminescence: A result of an electrochemical reaction

Luminescence is "cold light" that can be emitted at normal and lower temperatures.

Fluorescence and **Photoluminescence** are **luminescence** where the energy is supplied by electromagnetic radiation

FIREFLIES AND GLOW-WORMS

Fireflies and glow-worms (their larvae) are the best-known examples of **bioluminescent** creatures.

They use a complex reaction to make light from a pair of chemicals called luciferin and luciferase stored in their tails.

Bioluminescence is a special kind of chemoluminescence that happens inside living things.

MULTIPLEX (BIOPLEX) OR CYTOKINES ANALYZER

Cytokines are a large group of proteins, peptides or glycoproteins that are secreted by specific cells of immune system.

Cytokines are a category of signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis.

METHODOLOGY

1. Similar to ELISA, a majority of assays are designed according to a capture sandwich immunoassay format.

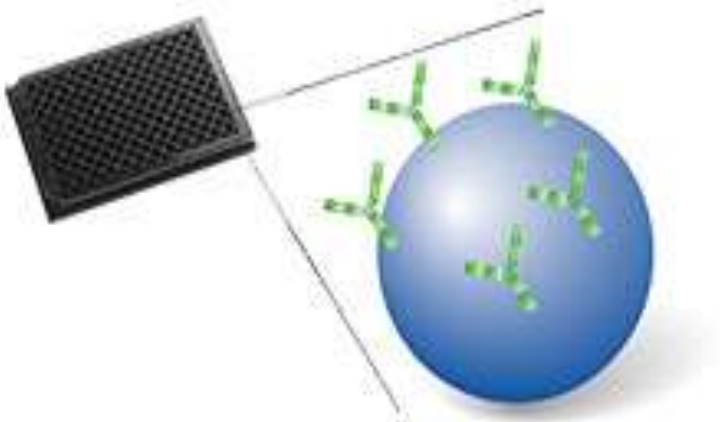
2. Briefly, the capture antibody-coupled beads are first incubated with antigen standards or samples for a specific time.

3. The plate is then washed to remove unbound materials, followed by incubation with biotinylated detection antibodies.

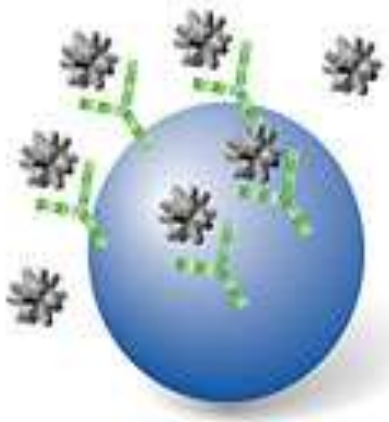
4. After washing away the unbound biotinylated antibodies, the beads are incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE).

5. Following removal of excess SA-PE, the beads are passed through the array reader, which measures the fluorescence of the bound SA-PE

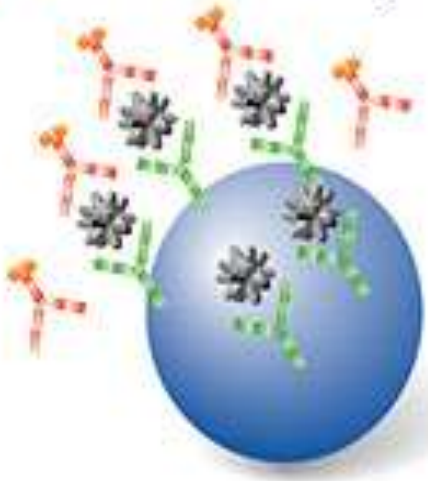
Step One
Dispense capture beads



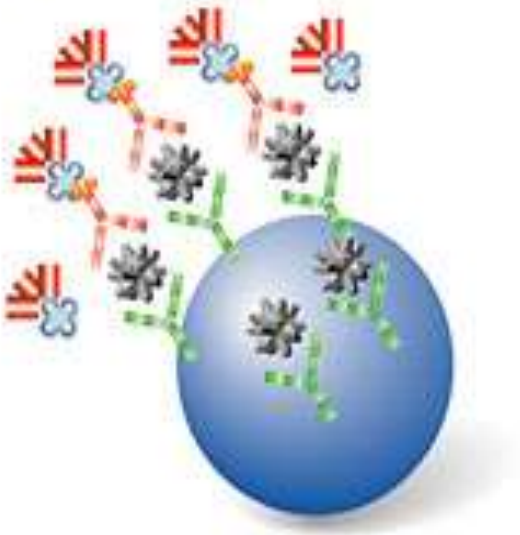
Step Two
Add samples



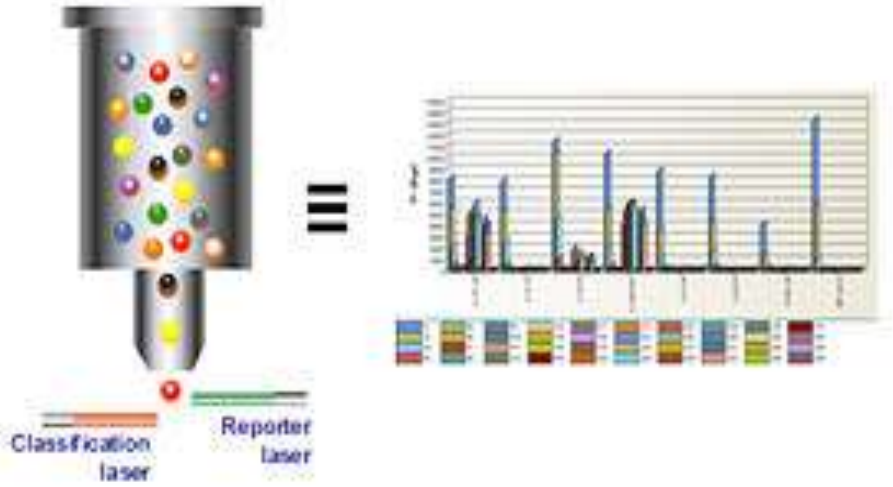
Step Three
Add detection antibody



Step Four
Add reporter dye

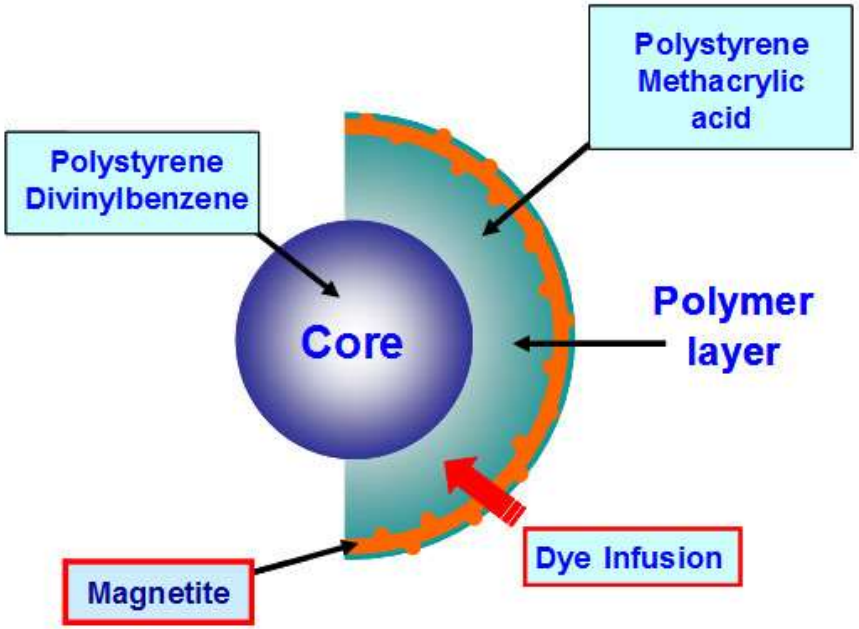


Step Five
Fluorescent sorting and data reduction



MULTIPLEX ASSAY BEADS

- The substrate for the antibody sandwich is the bead.
- Bead characteristics define instrument compatibility and workflow and can be classified into two basic categories, nonmagnetic and magnetic.
- The nonmagnetic beads are smaller in size (5.6 µ) and are used with the vacuum workflow; they are not compatible with certain instruments that utilize magnets for imaging purposes.



- Nonmagnetic beads utilize a vacuum workflow requiring filter plates and vacuum filtration to wash the beads.
- Magnetic beads are coated with magnetite and are therefore larger in size (6.5 µ); they can be used with the magnetic workflow as well as vacuum workflows.
- These magnetic beads (6.5 µ) are compatible with all currently available life science instruments from any Luminex partner.
- In the magnetic workflow the beads are washed in the well with dispense and aspiration washing.

IN SITU HYBRIDIZATION (ISH)

1. In situ hybridization was invented by Joseph G. Gall. In suite hybridization is biological assay (like ELISA, PCR) for molecular diagnosis.

2. ***In situ* hybridization (ISH)** is a powerful technique for localizing specific nucleic acid targets within fixed tissues and cells, allowing you to obtain information about gene expression and genetic loci.

3. In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (in situ), or, if the tissue is small enough (e.g. plant seeds, Drosophila embryos), in the entire tissue (whole mount ISH), in cells and in circulating tumor cells (CTCs).

4. In situ hybridization is a powerful technique for identifying specific mRNA species within individual cells in tissue sections, providing insights into physiological processes and disease pathogenesis. However, in situ hybridization requires that many steps be taken with precise optimization for each tissue examined and for each probe used.

5. In situ hybridization is used to reveal the location of specific nucleic acid sequences on chromosomes or in tissues, a crucial step for understanding the organization, regulation and function of genes.

Applications of ISH

Prenatal test during pregnancy

- Conventional prenatal tests for chromosomal abnormalities such as Down Syndrome rely on analyzing the number and appearance of the chromosomes the karyotype.
- Molecular diagnostics tests like microarray comparative genomic hybridisation test a sample of DNA instead, and because of cell-free DNA in plasma, is less invasive.

Pharmacogenomics

- Some of a patient's s—slight differences in their DNA—can help predict how quickly they will metabolize particular drugs; this is called pharmacogenomics.
- For example, the enzyme CYP2C19 metabolizes several drugs, such as the anti-clotting agent Clopidogrel, into their active forms. Some patients possess polymorphisms in specific places on the 2C19 gene that make poor metabolisers of those drugs; physicians can test for these polymorphisms and find out whether the drugs will be fully effective for that patient

Pathogenomics

- Molecular diagnostics are used to identify infectious diseases such as chlamydia, influenza virus and tuberculosis; or specific strains such as H1N1 virus.

Types of ISH

Today there are two basic ways to visualize your RNA and DNA targets *in situ*— fluorescence (FISH) and chromogenic (CISH) detection.

Chromogenic *in situ* hybridization (CISH) enables you to gain genetic information in the context of tissue morphology.

Fluorescence *in situ* hybridization (FISH) enables you to assay multiple targets simultaneously and visualize co-localization within a single specimen.

Characteristics inherent in each method of detection have made FISH and CISH useful for very distinct applications.

While both use a labeled, target-specific probe that is hybridized with the sample, the instrumentation used to visualize the samples is different for each method.

FLUORESCENCE IN SITU HYBRIDIZATION [FISH]

- In situ hybridization is the method of localizing/ detecting specific nucleotide sequences in morphologically preserved tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe against the sequence of interest.
- If nucleic acids are preserved in a histological specimen, then it can be detected by using a complementary probe

Principle

- Principle is same as that of In Situ Hybridization
- Use of a fluorescent labeled probe differentiates ISH & FISH
- FISH is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes

FISH: Biological Application

- FISH - a process which vividly paints chromosomes or portions of chromosomes with fluorescent molecules and Identifies any chromosomal abnormalities
- Aids in gene mapping, toxicological studies, analysis of chromosome structural aberrations, and ploidy determination
- Used to identify the presence and location of a region of DNA or RNA within morphologically preserved chromosome preparations, fixed cells or tissue sections
- This means you can view a segment or entire chromosome with your own eyes was often used during M phase but is now used on I phase chromosomes as well
- Advantage: less labor-intensive method for confirming the presence of a DNA segment within an entire genome than other conventional methods like Southern blotting

Application of FISH

1. Detection of high concentrations of base pairs. e.g.: Mouse metaphase preparation stained with DAPI (a non-specific DNA binding dye with high affinity for A-T bonds)

2. Centromere regions stained brighter - means they are rich in A-T bonds

3. Also used in germ cell or prenatal diagnosis of conditions such as aneuploidies

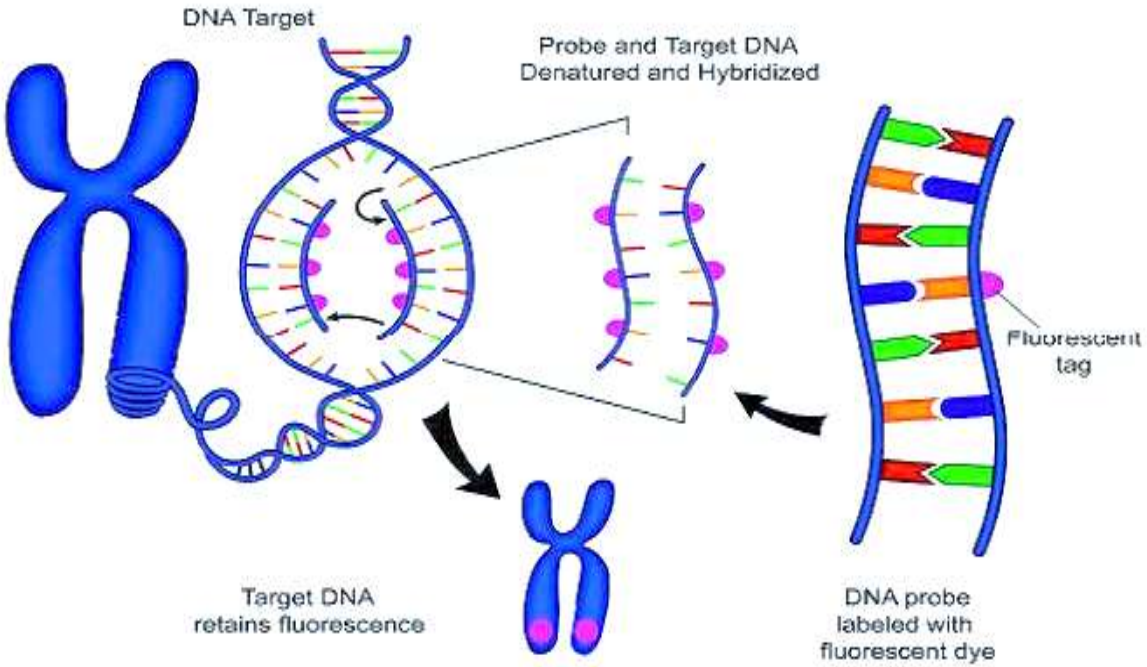
4. FISH can be used in the study of transgenic animals (eg: Polly)

5. Selective markers show if the human DNA was inserted successfully and pinpoint where the human DNA is

6. Transgenic Mouse

FISH Procedure

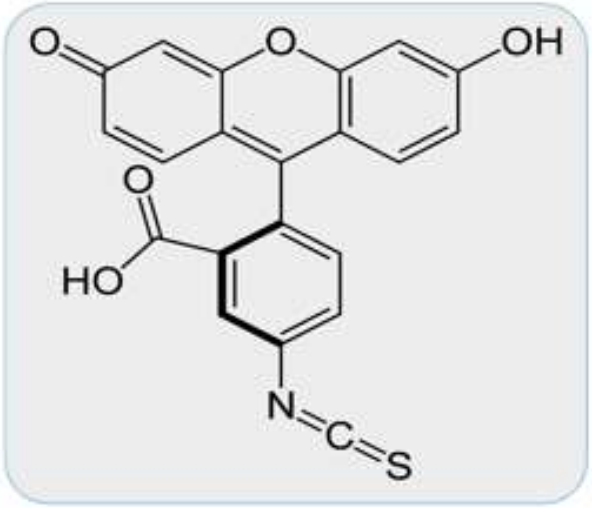
1. Denature the chromosomes
2. Denature the probe
3. Hybridization
4. Fluorescence staining
5. Examine slides or store in the dark



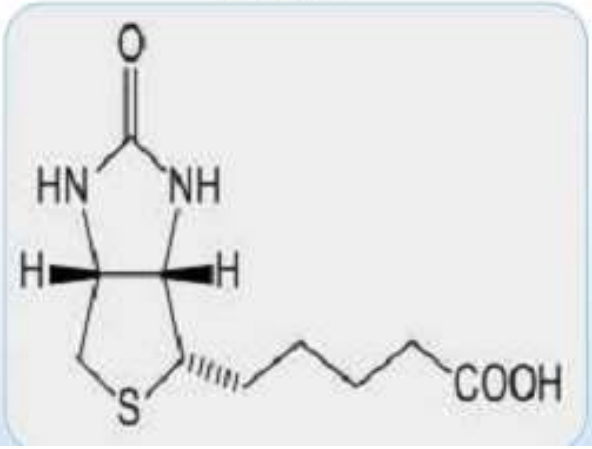
Schematic representation of FISH technique. A DNA probe is tagged with a fluorescent marker. The probe and target DNA are denatured, and the probe is allowed to hybridize with the target. The fluorescent tag is then detected with a fluorescent microscope.

Probes

Fluorescein



Biotin



- Complementary sequences of target nucleic acids
- Designed against the sequence of interest
- Probes are tagged with fluorescent dyes like biotin, fluorescein, Digoxigenin
- Size ranges from 20-40 bp to 1000bp

Types of Probes

- **Centromere probes**
  - Alpha and Satellite III probes
  - Generated from repetitive sequences found in centromeres
  - Centromere regions are stained brighter
- **Telomere**
  - Specific for telomeres
  - Specific to the 300 kb locus at the end of specific chromosome
- **Whole chromosome**
  - Collection of probes that bind to the whole length of chromosome
  - Multiple probe labels are used
  - Hybridize along the length of the chromosome
- **Locus**
  - Deletion
  - Translocation probes
  - Gene detection & localization probes
  - Gene amplification probes

Denaturation & Hybridization

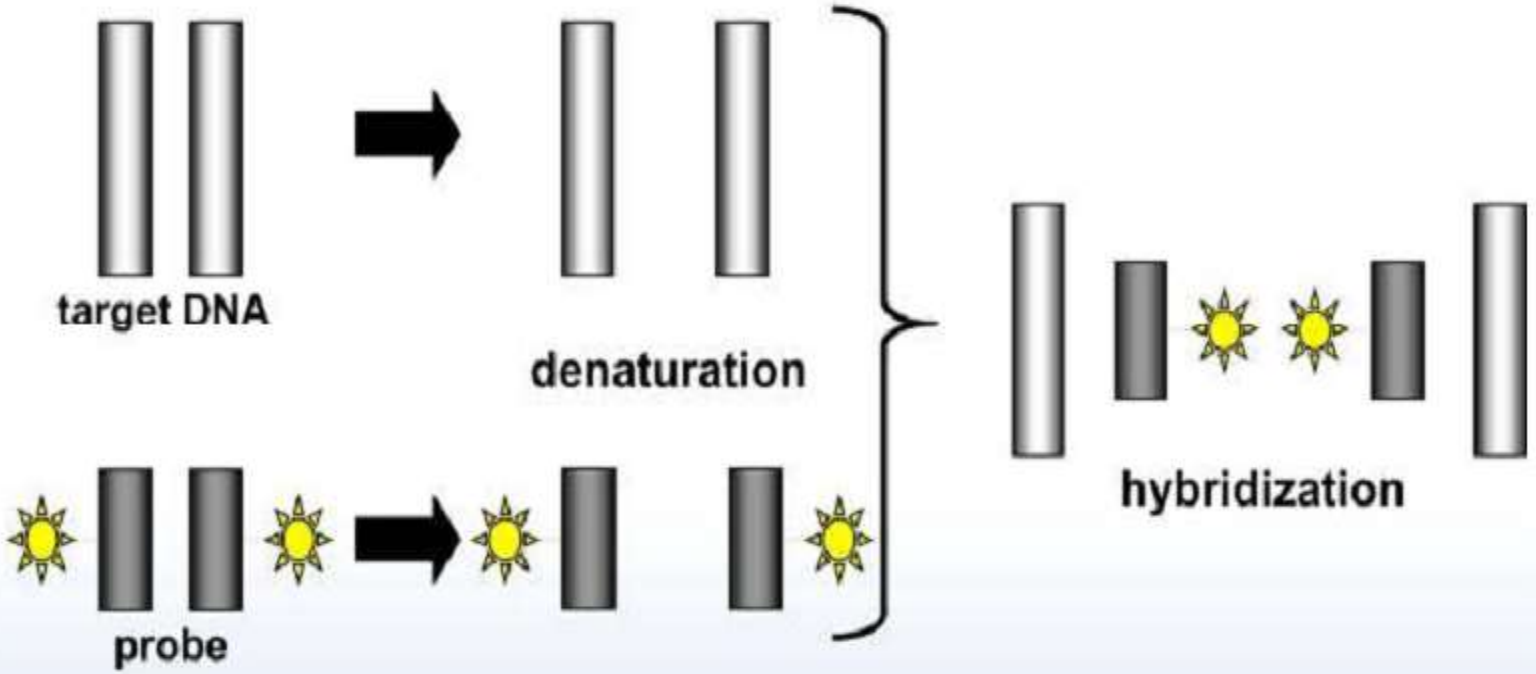
Denaturation

- Either by heat or alkaline method
- A prerequisite for the hybridization of probe and target

Hybridization

- Formation of duplex between two complementary nucleotide sequences
- Can be between
  - DNA-DNA
  - DNA-RNA
  - RNA-RNA

Hybridization



Detection & Visualization

Detection

- Direct labelling:
  - Label is bound to the probe
  - Less sensitive
- Indirect labelling:
  - Require an additional step before detection
  - Probe detected using antibodies conjugated to labels like Alkaline phosphatase
  - Results in amplification of signal

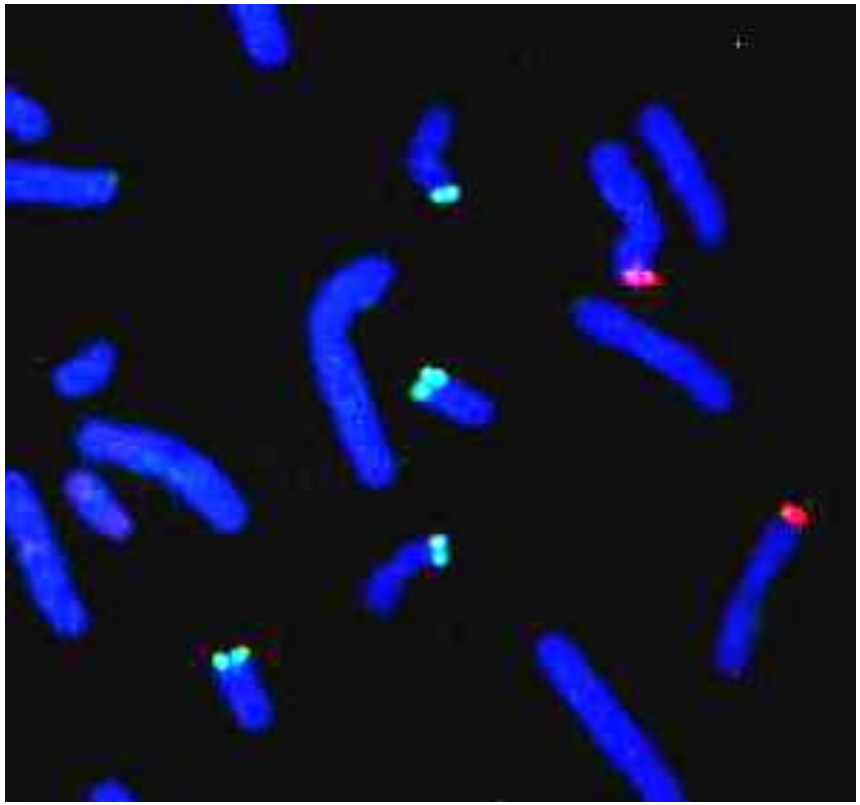
Hybridization

- Fluorescent probe attaches to the target sequence during hybridization
- This is visualized through a microscope with appropriate filters

Diagnostic Applications of FISH

- Prenatal diagnosis
- Cancer diagnosis
- Molecular cytogenetic of birth defects and mental retardation
- The identification of specific chromosome abnormalities
- The characterization of marker chromosomes
- Interphase FISH for specific abnormalities in cases of failed
- Cytogenetic
- Monitoring the success of bone marrow transplantation

FISH and Telomeres



Telomeric probes define the terminal boundaries of chromosomes (5’ and 3’ ends)

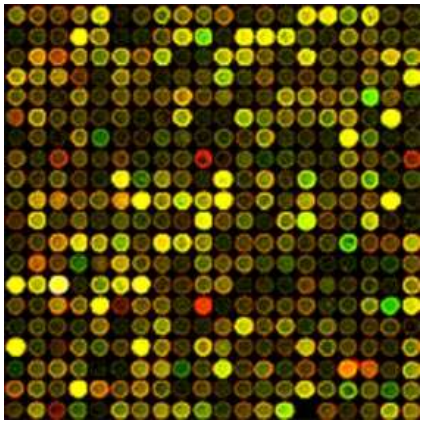
Used in research of chromosomal rearrangements and deletions related to cell aging or other genetic abnormalities

Special telomeric probes specific to individual chromosomes have been designed

Probe is based on the TTAGGG repeat present on all human telomeres

Application in cytogenetic - can detect submicroscopic deletions and cryptic translocations of genes associated with unexplained mental retardation and miscarriages

MICROARRAY



- Microarrays are sets of miniaturized chemical reaction areas that may be used to test DNA fragments, antibodies, or proteins.
- Each reaction area or spot is having immobilised target which is hybridised with complimentary probe present in the testing sample.

VARIOUS TYPES OF MICROARRAYS ARE :

- **DNA microarrays,** such as cDNA microarrays, oligonucleotide microarrays and SNP microarrays
- **MMChips,** for surveillance of microRNA populations
- **Peptide microarrays,** for detailed analyses or optimization of protein-protein interactions
- **Tissue microarrays**
- **Cellular microarrays** (also called transfection microarrays)
- **Chemical compound microarrays**
- **Antibody microarrays**
- **Carbohydrate arrays (glycoarrays)**
- **Phenotype microarrays**

DNA MICROARRAY / DNA CHIP

Bio chip is a small rectangular solid surface that is made of glass or silicone. Short DNA or RNA targets are anchored to its surface. (no. may vary from 10-20 to hundreds of thousands)

Each known gene occupies a particular “spot” on the chip.

The DNA sample which is to be analysed is amplified by PCR, then DNA is labelled with fluorescent tag and then loaded on chip

Hybridization occurs between sample DNA and target, allowing thousands of hybridization reactions to occur at the same time.

Complete sequence matching result in bright fluorescence and even a single base mismatching result in a dimmer fluorescence signal.

The solid surface of Biochip

Substrates for arrays are usually silicon chips or glass microscope slides.

Glass is a readily available and inexpensive support medium that has a relatively homogeneous chemical surface whose properties have been well studied and is amenable to chemical modification using very versatile and well developed silanization chemistry.

The two-dimensional surface is typically prepared by treating the glass or silicon surface with an amino silane which results in a uniform layer of primary amines or epoxides.

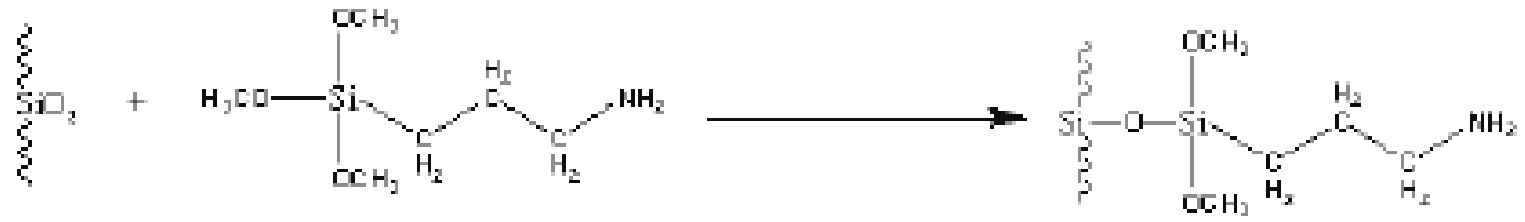


Figure 1. Surface modification. Amino modification with 3-aminopropyltrimethoxysilane.

SURFACE MODIFICATION AND ADVANTAGES

These modifications results in several advantages:

1. The linkages are chemically stable,
2. Sufficiently long to eliminate undesired steric interference from the support, and
3. Hydrophilic enough to be freely soluble in aqueous solution and not produce non-specific binding to the support.

Once these modifications have activated the surface, the efficiency of attaching the oligonucleotides depends largely on the chemistry used and how the oligonucleotide targets are modified.

Fabrication of the surface with oligonucleotide

Fabrication is done by two methods:

1. Spotted microarray
2. In situ synthesised array / *oligonucleotide microarrays*

SPOTTED MICROARRAY

The probes are synthesized prior to deposition on the array surface and are then "spotted" onto glass.

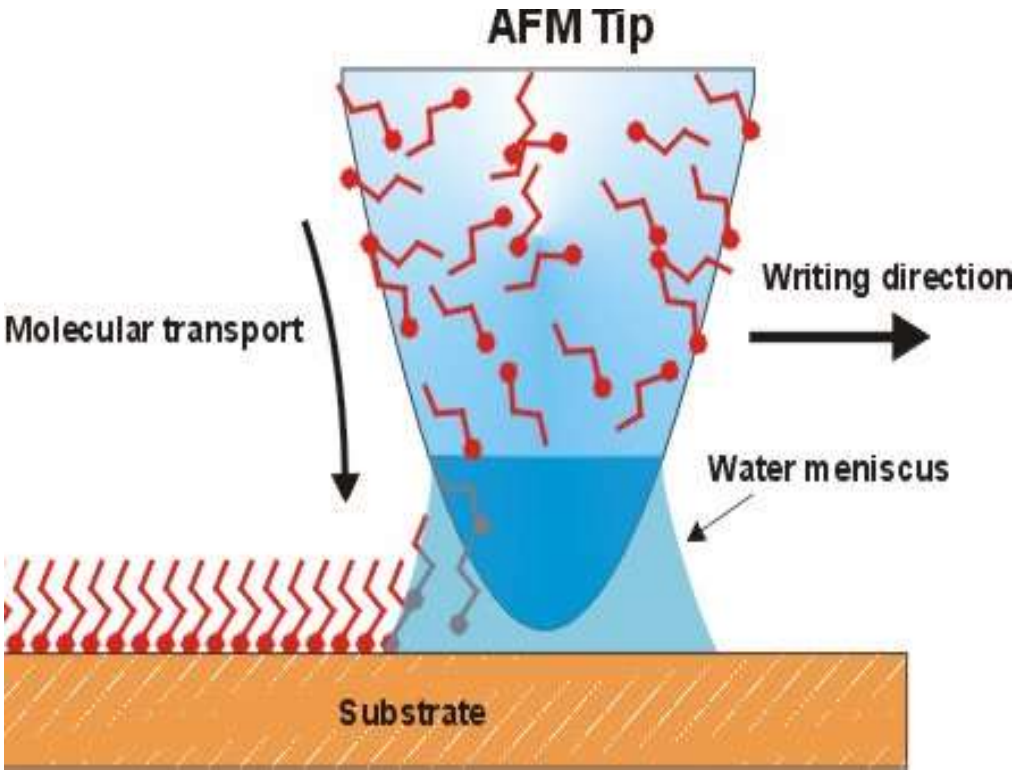
Spotting is done by a technique which utilises atomic force microscopy; known as Dip Pen Nanolithography

In *spotted microarrays*, the probes can be -

i. Oligonucleotides

ii. cDNA or

iii. Small fragments of PCR products that correspond to mRNAs.



ADVANTAGES

- This technique is used by research scientists around the world to produce "in-house" printed microarrays from their own labs.
- These arrays may be easily customized for each experiment, because researchers can choose the probes and printing locations on the arrays, synthesize the probes in their own lab (or collaborating facility), and spot the arrays.
- This provides a relatively low-cost microarray that may be customized for each study, and avoids the costs of purchasing often more expensive commercial arrays that may represent vast numbers of genes that are not of interest to the investigator.
- In-house spotted microarrays may not provide the same level of sensitivity compared to commercial oligonucleotide arrays, possibly owing to the small batch sizes and reduced printing efficiencies when compared to industrial manufactures of oligo arrays.

OLIGONUCLEOTIDE ARRAY

Oligonucleotide arrays are produced by printing short oligonucleotide sequences designed to represent a single gene or family of gene splice-variants.

Printing is done by synthesizing this sequence directly onto the array surface instead of depositing intact sequences as done in spotted microarray.

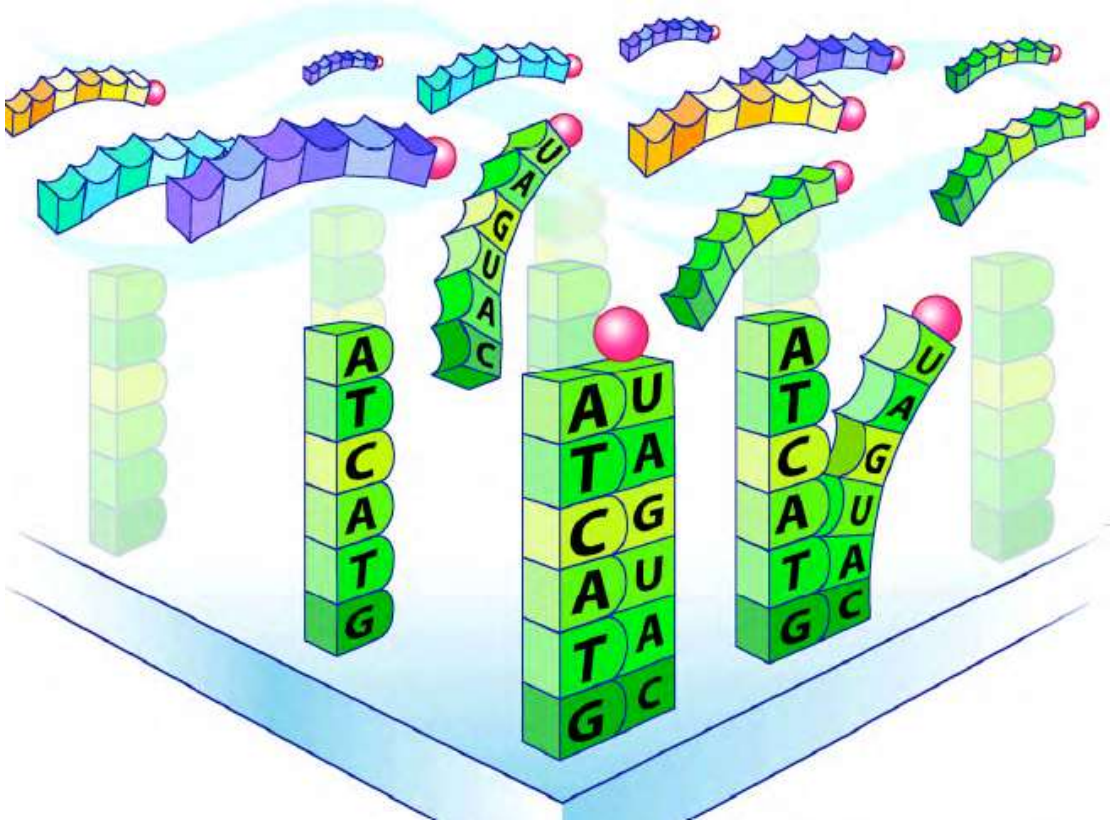
The technique used to produce oligonucleotide arrays include photolithographic synthesis (Affymetrix) on a silica substrate where light and light-sensitive masking agents are used to "build" a sequence one nucleotide at a time across the entire array.

Fluorescence tagging of DNA sample

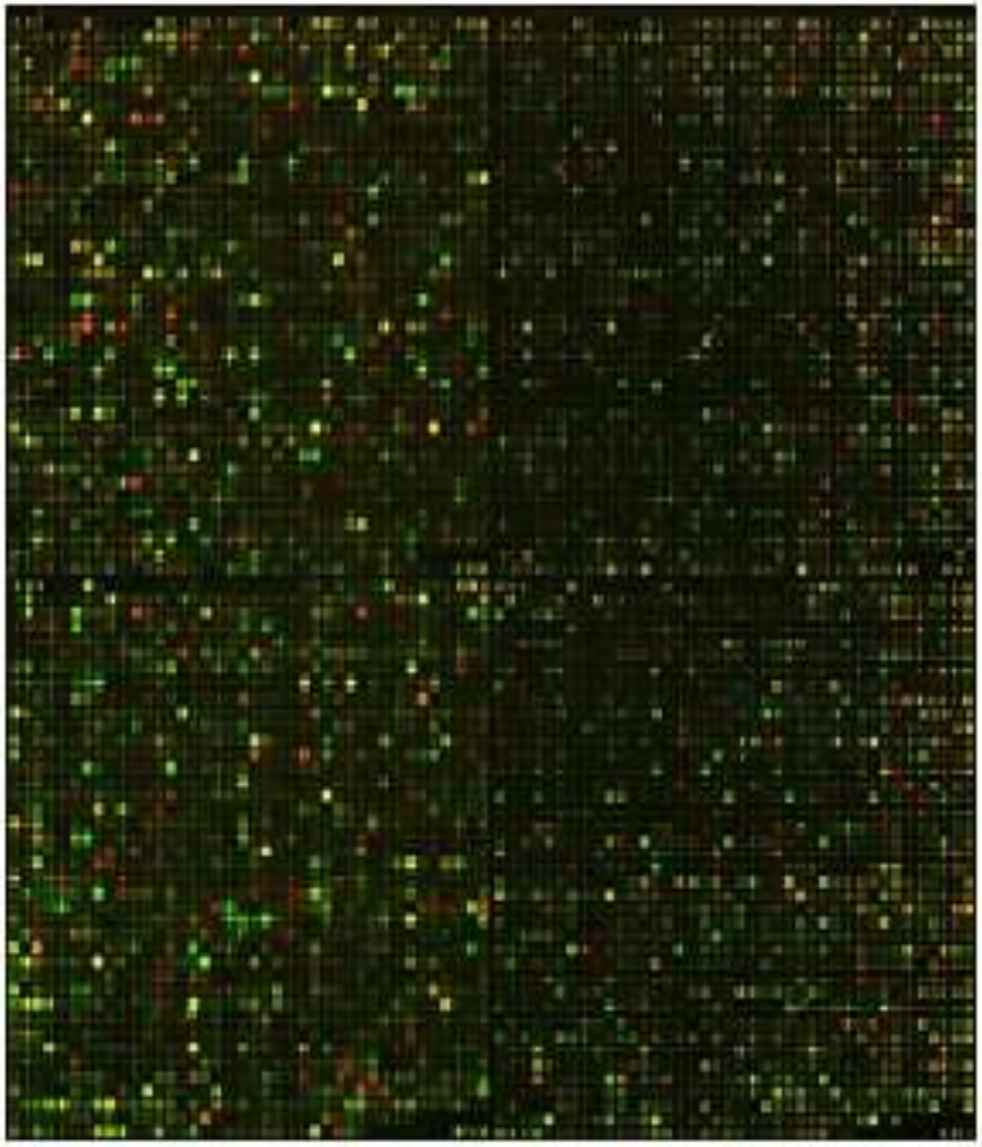
Fluorescent dyes commonly used for cDNA labeling include Cy3, which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and Cy5 with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum).

Array analysis

Hybridization is analysed by Laser Induced fluorescence (LIF).



use of LIF in data acquisition



reads
: Colour
: Intensities

this requires very sophisticated computer analysis

SINGLE CHANNEL MICROARRAY

- In *single-channel microarrays* or *one-color microarrays*, the arrays provide intensity data for each probe or probe set indicating a relative level of hybridization with the labelled target.

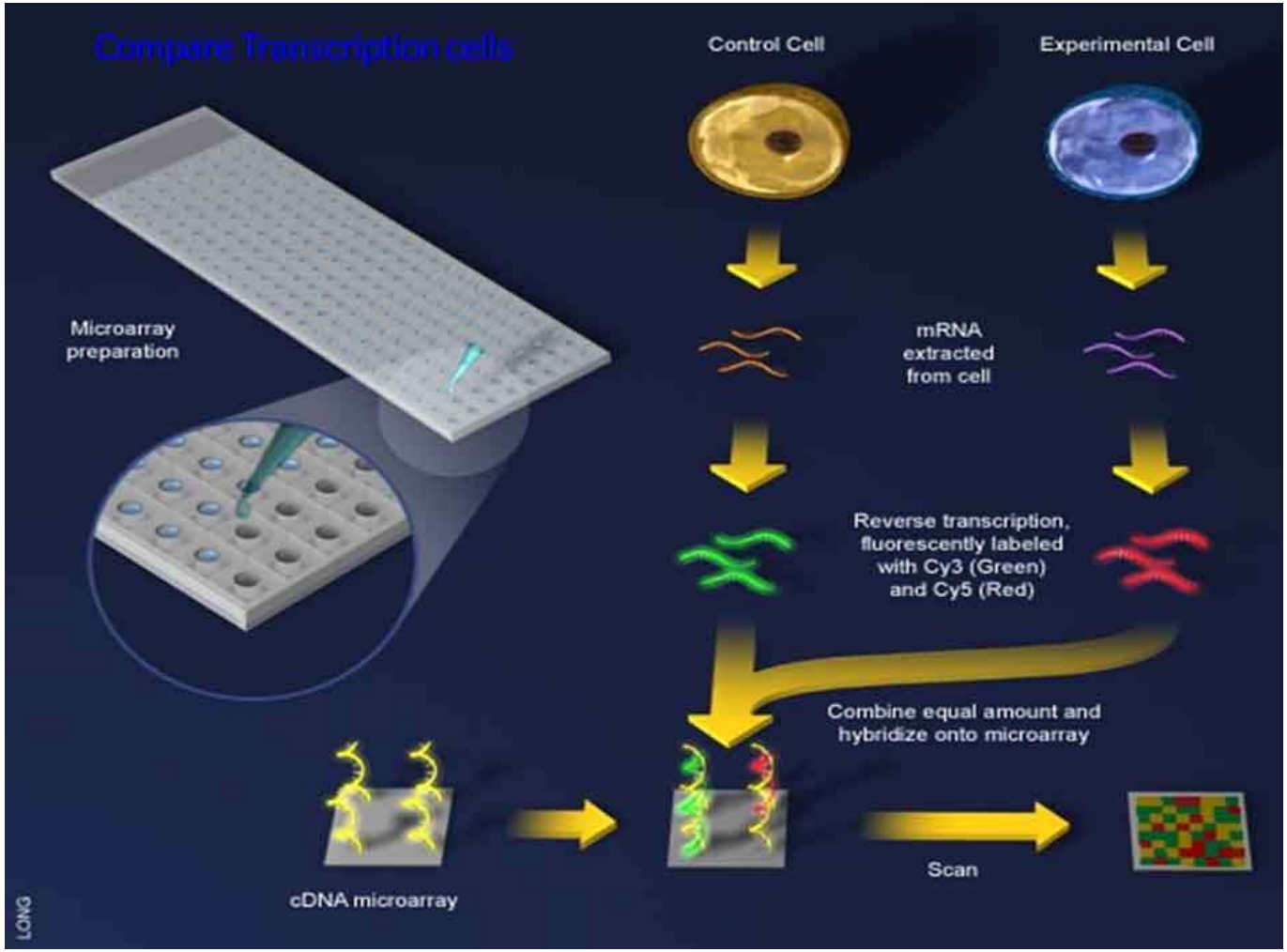
- One strength of the single-dye system lies in the fact that an aberrant sample cannot affect the raw data derived from other samples, because each array chip is exposed to only one sample (as opposed to a two-colour system in which a single low-quality sample may drastically impinge on overall data precision even if the other sample was of high quality).

- Another benefit is that data are more easily compared to arrays from different.

- A drawback to the one-color system is that, when compared to the two-colour system, twice as many microarrays are needed to compare samples within an experiment.

TWO CHANNEL MICROARRAY

Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes.



APPLICATION OF DNA MICROARRAY

Analysis of Gene Expression

1. Examining expression during development or in different tissues
2. Comparing genes expressed in normal vs. diseased states
3. Analyzing response of cells exposed to drugs or different physiological conditions

Monitoring Changes in Genomic DNA

1. Identify mutations and Identifying polymorphisms (SNPs)
2. Examining genomic instability such as in certain cancers and tumors (gene amplifications, translocations, deletions)
3. Diagnosis: chips have been designed to detect mutations in p53, HIV, and the breast cancer gene BRCA-1

Applications in Medicine

1. Inferring regulatory networks
2. Pathogen analysis (rapid genotyping)
3. Staging of leukemias

Applications in Drug Discovery

1. Identify appropriate molecular targets for therapeutic intervention (small molecule / proteins)

2. Monitor changes in gene expression in response to drug treatments (up / down regulation)

3. Analyze patient populations (SNPs) and response

Targeted Drug Treatment

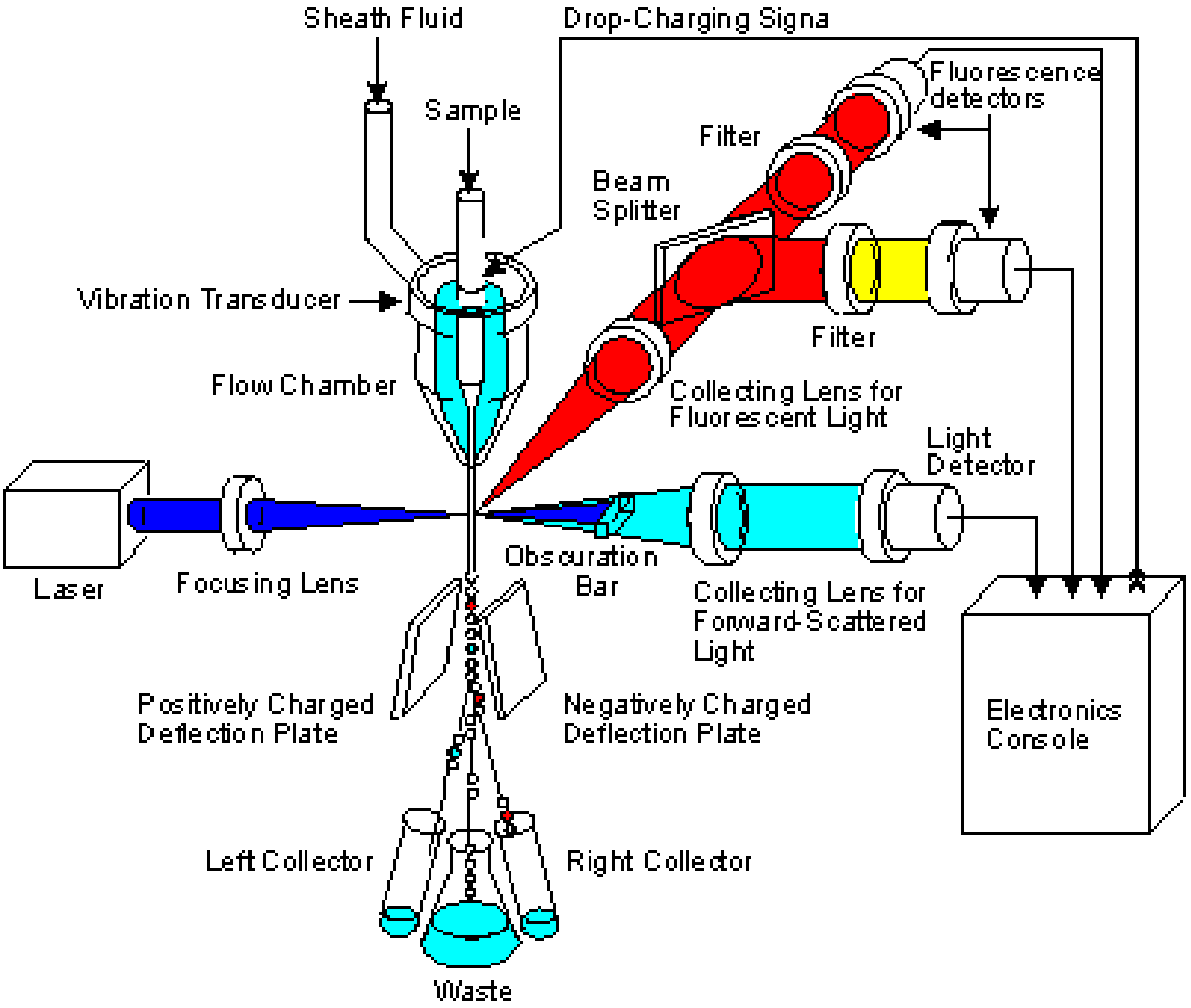
1. Pharmacogenomics: individualized treatments
2. Choosing drugs with the least probable side effects

FLOW CYTOMETRY / FLUORESCENCE FLOW CYTOMETRY

- Flow cytometry is a technology used for rapid cell count analysis and forms the pillar for a basic blood count test.
- This is basic technology supporting any pathological laboratory involved in providing blood count services.
- However, in recent times, along with enumerating basic blood cell count, technological advancements have introduced the possibility to include wide variety of rare cell population enumerations as well, which end up increasing the diagnostic value of the report generated.
- For HIV detection, the blood cells are generally tagged using fluorescent labeled antibodies specific for CD3, CD4 and CD8 enumeration. In case of complete blood count analysis, the blood cells are stained differentially and analyzed.

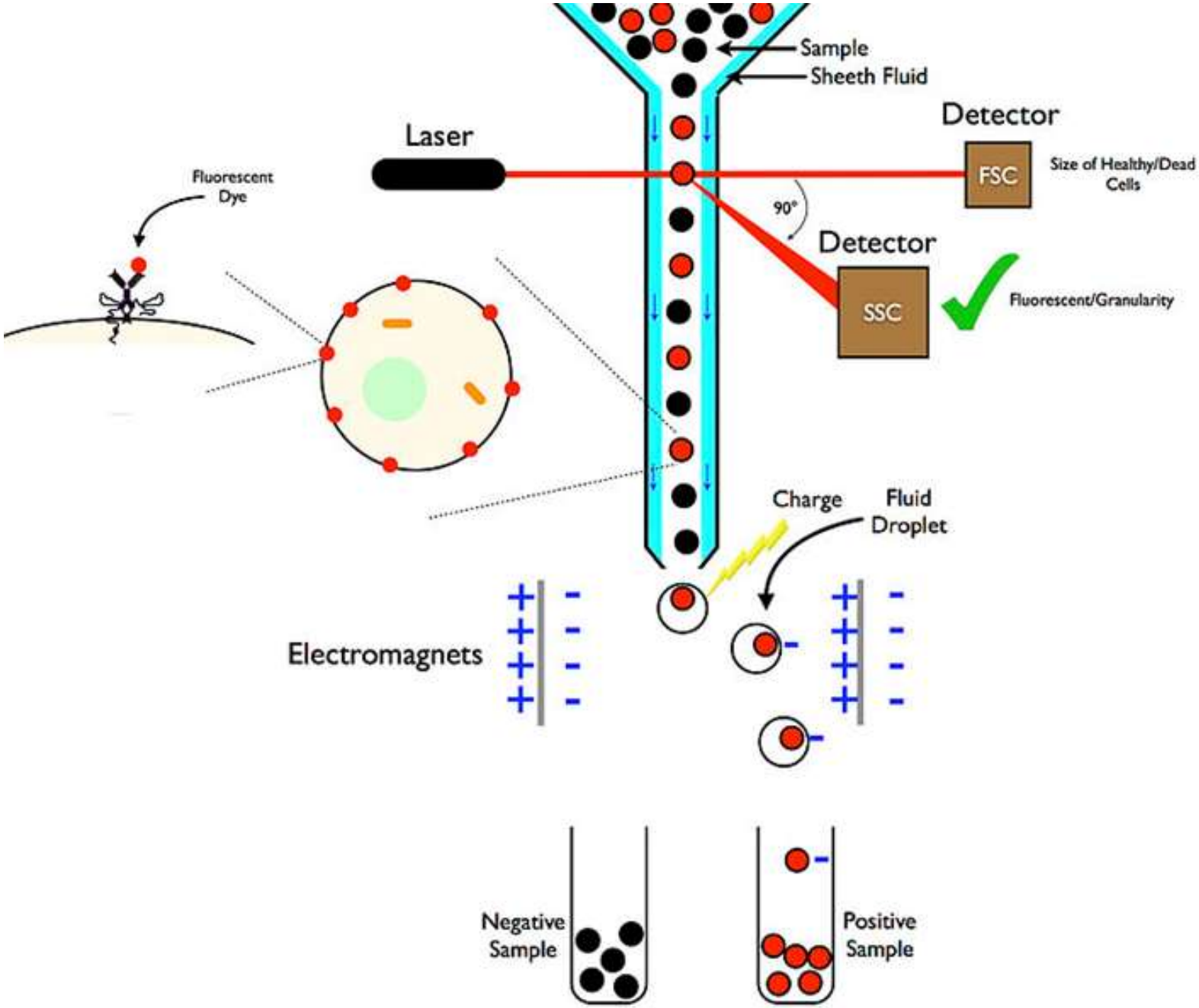
PRINCIPLE OF FLOW CYTOMETRY / FLUORESCENCE FLOW CYTOMETRY

- Flow cytometry involves enumeration of cells in a liquid flow. The cells are either tagged with fluorescent antibodies or are stained and suspended in a liquid stream within a flow cell.
- The pressure of the sheath buffer ensures the cells flow in a narrow stream toward the laser source, and the beam hits only one single cell at a time.
- The incident light on the cell is then scattered forward as well as sideways. This scattered light is captured by the optics in the analyzer and directed to the detector.
- The cells are analyzed on the basis of their shape, size as well as internal complexity


Sheath Fluid
Drop-Charging Signa
Sample
Fluorescence detectors
Filter
Beam Splitter
Vibration Transducer
Filter
Flow Chamber
Collecting Lens for Fluorescent Light
Light Detector
Laser
Focusing Lens
Obscuration Bar
Collecting Lens for Forward-Scattered Light
Electronics Console
Positively Charged Deflection Plate
Negatively Charged Deflection Plate
Left Collector
Right Collector
Waste

FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

- Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry.
- It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell.
- It is a useful scientific instrument as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.
- The technique was expanded by Len Herzenberg, who was responsible for coining the term FACS. Herzenberg won the Kyoto Prize in 2006 for his seminal work in flow cytometry


Sample
Sheeth Fluid
Detector
Laser
FSC
Size of Healthy/Dead Cells
Fluorescent Dye
90°
Detector
SSC
Fluorescent/Granularity
Charge
Fluid Droplet
Electromagnets
Negative Sample
Positive Sample

FACS WORKING PRINCIPLE

1. The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter.

2. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet.

3. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured.

4. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream.

5. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream.

6. The stream is then returned to neutral after the droplet breaks off.

References

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