AMERICAN JOURNAL OF PHARMTECH RESEARCH

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Review On: High-Throughput Screening Is An Approach To Drug Discovery

Ashok A. Hajare[*1], Sachin S. Salunkhe[2], Sachin S. Mali[1], Sonali S. Gorde[3], Sameer J. Nadaf[1], Sachin A. Pishawikar [2]

1 Department of Pharmaceutical Technology, Bharati Vidyapeeth College of Pharmacy, Near Chitranagari, Kolhapur, 416 013, Maharashtra, India.

2 Department of Quality Assurance, Bharati Vidyapeeth College of Pharmacy, Near Chitranagari, Kolhapur, 416 013, Maharashtra, India.

3 Department of Pharmaceutics, Modern College of Pharmacy (For Ladies) Moshi, Pune-412105, Maharashtra, India.

ABSTRACT

High-Throughput Screening (HTS) is an approach to drug discovery that has gained widespread popularity over the last two decades and has become a standard method for drug discovery in the pharmaceutical industry. Progress from traditional one-compound-at-a time approach, low throughput screening to high throughput screening involving fully automated robotic systems, enables testing of large numbers of compounds daily for different activities in miscellaneous areas of biology. HTS reveals screening of more than 100,000 samples per day. Compared to traditional drug screening methods, HTS is characterized by its simplicity, rapidness, low cost, and high efficiency. Identification of good hits using HTS can minimize the time span of drug discovery noticeably. However, synthetic chemistry for lead optimization and the low throughput of secondary assays for defining the crucial pharmacological properties of active compounds, limits the overall rate of identification of candidate molecules for clinical evaluation. Coupling of compound library with wide chemical diversity along with HTS shows massive drug discovery potential, but to be successful screening technique it depends on several factors. These include the number and quality of validated targets, the number and diversity of compounds in the collections, and the ability to screen these in a timely and cost effective manner using robust informative assays. In this review we have discussed the types of HTS assays, assay miniaturization automation and different detection techniques like fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), homogeneous time resolved fluorescence (HTRF) etc.

Keywords: HTS, FRET, FP, HTRF, Cell based assays, Statistics.

*Corresponding Author Email: aahajare@rediffmail.com

Received 09 December 2013, Accepted 17 December 2013

Please cite this article in press as: Hajare AA. *et al.*, Review On: High-Throughput Screening Is An Approach To Drug Discovery. American Journal of PharmTech Research 2014.

INTRODUCTION

In this review we have discussed the types of HTS assays, assay miniaturization automation and different detection techniques like fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), homogeneous time resolved fluorescence (HTRF) etc. High-throughput screening methods are also used to characterize metabolic, pharmacokinetic and toxicological data about new drugs. Discovery of a new drug molecule from primary idea to development of a finished product is an intricate process which can take up to 12–15years and cost more than approximately $1 billion.[1] High-throughput screening (HTS) is a method for scientific experimentation especially used in drug discovery and may be applicable to the fields of biology and chemistry. HTS is a process to accelerate drug discovery, which involves a brute force approach where tens of thousands of compounds (Compound libraries) are tested against a particular target daily using a quantitative bioassay via the use of automation, miniaturized assays, micro fluidic chips, sub nano litre dispensing, fluorescence, large-scale data analysis.[2-5] Solid Phase Organic Synthesis has become a powerful tool for the preparation of compound libraries used for screening, which may have million of compounds, selected for drug- like characteristics such as solubility, partition coefficient, molecular weight, and number of hydrogen bond donors/ acceptors.[6,7] Generally drug discovery involves searching and testing of drug candidate over a preselected therapeutic targets.[8] For this purpose to accelerate drug discovery a target-focused library involving collections of compounds which are designed to interact with an individual protein target or, frequently, a family of related targets (such as kinases, voltage-gated ion channels, serine/ cysteine proteases) is useful.[9] Primary advantage of this is that, higher rates of hit formation are observed compared to screening of diverse sets.[10]

Compared to traditional drug screening methods, HTS is characterized by its simplicity, rapidness, low cost, and high efficiency. Generally, HTS involves an automated operation-platform, modern robotics, highly sensitive detection methods, sophisticated control software, specific screening model (in vitro), an abundant components library, advanced liquid handling and a data acquisition and processing system.[11,12] Now it has become possible to screen more than 1,00,000 samples per day due to Several technologies such as fluorescence, nuclear-magnetic resonance, affinity chromatography, surface plasma on resonance and DNA microarray etc.[13] HTS is an effectual way of reducing a prohibitively large number of diverse chemical starting points to a few promising structures that can be explored in more depth. An ideal HTS assay is simple, homogeneous, robust, and reproducible.[14] The purpose of HTS is to identify the

hits, active on the target and that can then be further converted by chemical optimization to a genuine lead (with appropriate potency and selectivity) which emerges as candidate for clinical development. The hits generated during HTS can be used as the starting point for a drug discovery effort. Typically, hits are refined through medicinal chemistry and lower throughput assays before entering the clinic.[7]

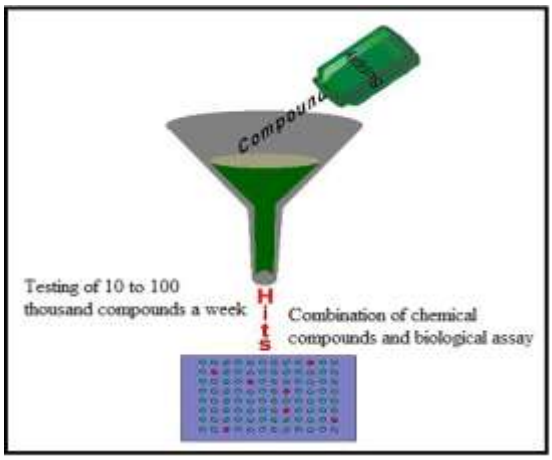

Figure 1: Formation of Hit used in HTS.

High throughput screening:

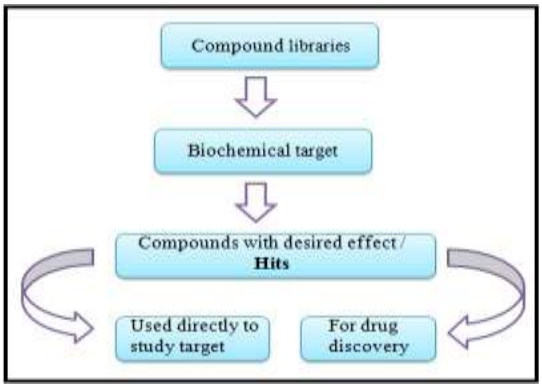

Figure 2: High throughput screening.

The mechanism-based approach which corresponds to the target-based approach screens compounds with a specific mode of action and provides improved drugs.[11] Precise focus of HTS on single mechanism contributes to its highly effectual nature for identification of target specific compounds. HTS assays found to be applicable for screening of different types of libraries, including combinatorial chemistry, genomics, protein, and peptide libraries.[3] Basically HTS is a process of screening and assaying large number of biological modulators and effectors against selected and specific targets.[15]

The main objective of this technique is to speed up the drug discovery process by screening the large compound libraries with a speed which may exceed a few thousand compounds per day or per week. For any assay or screening by HTS to be successful numerous steps like target

identification, reagent preparation, compound management, assay development and high-throughput library screening should be carried out with extreme care and precision.[16] Methods commonly followed are: firstly selecting the target. Currently there are about 500 targets being used by companies. Of these, cell membranes receptors, mostly G-protein coupled receptors, comprise the largest group (45% of the total), Enzymes make up the next largest group (28%), followed by hormones (11%), unknowns (7%), ion-channels (5%), nuclear receptors (2%), and finally DNA (2%).[16] in recent studies, pharmaceutical companies mainly look for for compounds that interfere or modulate the function of GPCRs.[17]

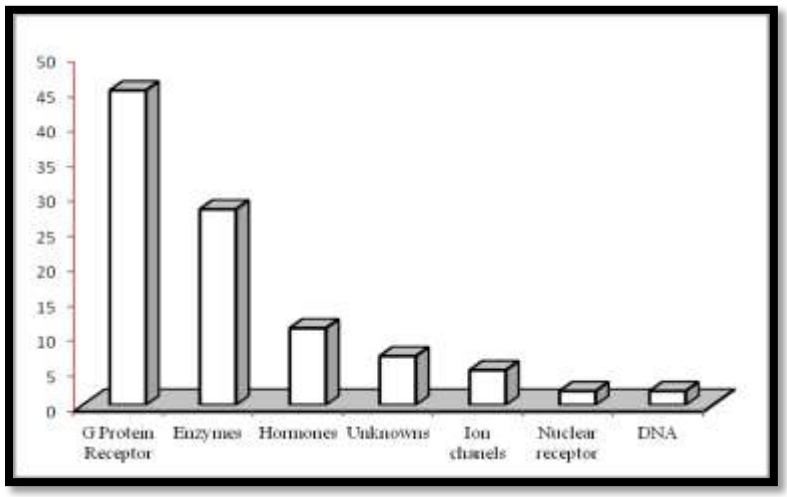

Figure 3: Targets in HTS.

HTS plays an essential role in the drug discovery process. Coupling of compound library with wide chemical diversity along with HTS shows massive drug discovery potential, but to be successful screening technique it depends on several factors. These include the number and quality of validated targets, the number and diversity of compounds in the collections, and the ability to screen these in a timely and cost effective manner using robust informative assays. Identification of good hits using HTS can minimize the time span of drug discovery noticeably. However, synthetic chemistry for lead optimization and the low throughput of secondary assays for defining the crucial pharmacological properties of active compounds, limits the overall rate of identification of candidate molecules for clinical evaluation.

1. Screening formats:

As previously mentioned assays should be informative, robust, reliable, reproducible, fast, cost effective and should easy to perform. The primary goals of automation, miniaturization, and high throughput are to develop homogeneous assay formats and the use of high sensitivity detection techniques. Several non separation (homogeneous) or "mix-and measure" assay

technologies are widely used as it provide simplicity, and avoids steps which are time-consuming and difficult-to-automate, such as extraction, filtration, centrifugation, and washing. However 96-well filter plates provides a provision if filtration is required in a particular assay.[18] Non isotopic end points such as fluorescence and luminescence based detection techniques are prominently used due to increased sensitivity, flexibility in assay design, low cost and improved safety, although radiometric assays, scintillation proximity assays have played a major role in HTS.[19,11]

2. Miniaturization:

Miniaturization minimizes costs, prevent unnecessary depletion of valuable compound supplies, reduce the amounts of reagents used, and decrease the costs of disposing of assay waste by enabling the use of parallel sample processing and multiplexed detection modes. To general approach to further miniaturization of assay is to proceed with plate based system involving higher well densities and smaller well volumes; and highly sensitive technique to execute continuous flow assays.[19,20] Miniaturization involves shift from the standard 96-well plate to higher density microplate formats.

Until recently 96-well micro liter plate (maximum volume 200–250 µl)is used for HTS, use of 384 well plates and higher density plates have been reported.384-well micro plate (maximum volume 70–100 µl)is the first miniaturized format used successfully in HTS. Converting assays from 96 to 384-well formats offers the benefits like potential reduction of reagent (from 200 µl in a 96 well to 50 µl) and radioactivity usage, reduced disposal costs and increased throughput (Kim dyer).one 384 well plate is equivalent to four 96 well plates.HTS assay has also been performed in 864 well plates.[21]Ultra-High Throughput Screening of Two-Million-Member Combinatorial Compound Collection in a Miniaturized, 1536-Well Assay Format has also reported. Berg et al. carried out a Miniaturization of an Enzyme Assay (β-Galactosidase) in the 384- and 1536-Well Plate Format.[22] In another study maffia et. Al. performed the luciferase reporter gene assays in mammalian T cells using a 1,536-well plate format.[23] Mere et. al.carried out a (FRET)-based biochemical and cell-based assays in 3456-well Nano well trade mark assay plates.[24]Assays carried out in even higher density plates(9600-Well 0.2 Microliter) have been described, which shows similar results when compared with standard 96-well format. (Oldenburg et al.,1998).

Performing an assay using higher density plates (1536well or above) faces some technical problem. Such as sensitivity to final dimethyl sulfoxide concentration and the limitations

inherent in present day low volume liquid dispensing technologies, and require monitoring of evaporation.

Recently microfabricated fluidic devices has been widely utilised to perform biochemical and cell-based assays. It has beenfocus of ongoing research because micrometer sized channels requires, smaller volume of sample and reagents, while electroosmotic phenomenon provide control over small volume size and eliminate the need of pump and valve.[25-26] Microchips (10-100µm) are fabricated in fused-silica substrates using standard lithography followed by wet chemical etching and thermal bonding.[27] Starkey et. al. carried out Fluorogenic assay for b-glucuronidase using microchip based capillary electrophoresis. Hydrolysis of the conjugated glucuronide, fluorescein mono-β-D-glucuronide(FMG),in presence of β-glucuronidase liberated the fluorescein (fluorescent product), further detected by fluorescence.[28]

3. Automation and robotics:

Considerable degree of automation is essential to increase throughput above that achieved by conventional, manual techniques, and to reduce pipetting errors, speed up the plate preparation process and to ensure sample uniformity in a screening context, especially on higher density plates.[29]Automation of laboratory procedures, led to a significantly accelerated drug discovery process compared to the traditional one-compound-at-a time approach. Traditionally, an experienced organic chemist could synthesize and finalize approximately 50 compounds each year; however due certain automation more than 2000 compounds can be easily generated yearly.[30]Automation of liquid handling has significantly decreased the time and effort required for preparation of compound libraries, assay preparation and ultimately speed up overall screening. Laboratory automation and robotics implementation enables high-capacity and high-throughput (100 K-1 MM/day) screening of large "libraries" of compounds (>200 K-2 MM) in a few days or weeks.[31] Utilisation of Linear robotic system enables faster throughput and an industrial style approach to drug discovery screening. This differs from and has several advantages over “robo-centric” style of automation. Zymark Allegro Combo™ now provides a modular linear based approach which directly competes with the flexibility and price range of integrated “robo-centric”systems.[32] A new automated method for isolation of total RNA in a 96-well format is described in a new technical note. In this RNA Microprep Kit allows high throughput isolation of total RNA from small samples of cultured cells.[33] In another study Michael et al. developed quantitative HTS (qHTS) paradigm, which tests each library compound at multiple concentrations to construct concentration-response curves (CRCs) generating a comprehensive data set for each assay.[34]

Pharmaceutical companies are under stock market pressure to synthesise and screen a newer product and generate a lead very fastly, further followed by preclinical and clinical studies. So to domain market and produce newer drug faster automation has become essential tool in pharmaceutical industries.[35]

TYPES OF HIGH THROUGHPUT ASSAYS:

Assays mainly divided into biochemical and cell based assays. Biochemical assays are further divided into heterogeneous and homogeneous assays.

A. Homogeneous assay:

In homogeneous assay measurement are based on the distinct physical/chemical properties of analyte, or interaction between analyte and surrounding environment. It is a single step process; reagent may be added at single stage or in multiple steps. It only involves usual steps like fluid addition, incubation and reading. It can be coupled with different detection technique fluorescence, radiometric etc for HTS. Main advantage of homogeneous assay is its simplicity (Mix and read) because minimum step which ultimately contributes to the reduction of both cost and robotic complexity required for automation. Some interference occurs in measurement because it has been carried out in presence of other assay components. It have signal to background ratio less than 10.[36,37]

B. Heterogeneous assays:

Heterogeneous assays involves additional steps like filtration, centrifugation etc. that separates component(s) to be measured from the rest of component which may interfere in assay. This contributes to the high signal to background ratio. Due to higher steps it becomes complicated. Heterogeneous assay are performed mainly when homogeneous assay fails or high signal to background ration is required.[36,37]

1. Biochemical assays:

Biochemical assays are receptor, protein or enzyme based assays uses the particular target in a purified form. Biochemical assays are most frequently carried out using scintillation proximity assay (SPA),radiometric, colorimetric fluorescence detection techniques. Scintillation Proximity Assay is a technology whereby binding reactions can be assayed without the washing or filtration procedures normally used to separate bound from free fractions. Radioactive labels that emit electrons at about 10 µm in water are used to carry out assay.[38] SPA technique is usually preferred for all surface cell receptors due to high binding and low receptor density required.[39] Some techniques of biochemical assays are summarized below:

Fluorescence resonance energy transfer (FRET):

Resonance energy transfer is a non-radiative quantum mechanical process, involves distance dependant transfer of energy from an excited donor fluorophore to a suitable acceptor fluorophore. In this process, donor fluorophore absorbs the energy from incident light and transfer this energy to nearby acceptor molecule. One frequent pair fluorophores is a cyan fluorescent protein (CFP) – yellow fluorescent protein (YFP) pair, spectrally distinct variants of green fluorescent protein (GFP).[40]

Following conditions must pertain for an effective FRET assay:

i) There should be an overlap between fluorescence emission spectrum of the donor molecule and the absorption or excitation spectrum of the acceptor chromophore. The degree of overlap is called as spectral overlap integral (J).

ii) Donor and acceptor fluorophore must be close to each other(typically 1 to 10 nanometer).

iii) There should be significant difference in extent of quenching of the starting material and product.

iv) Transition dipole orientations of donor and acceptor must be approximately parallel.[41,42] (SANGEETA SAINI, Syed Arshad Hussain)

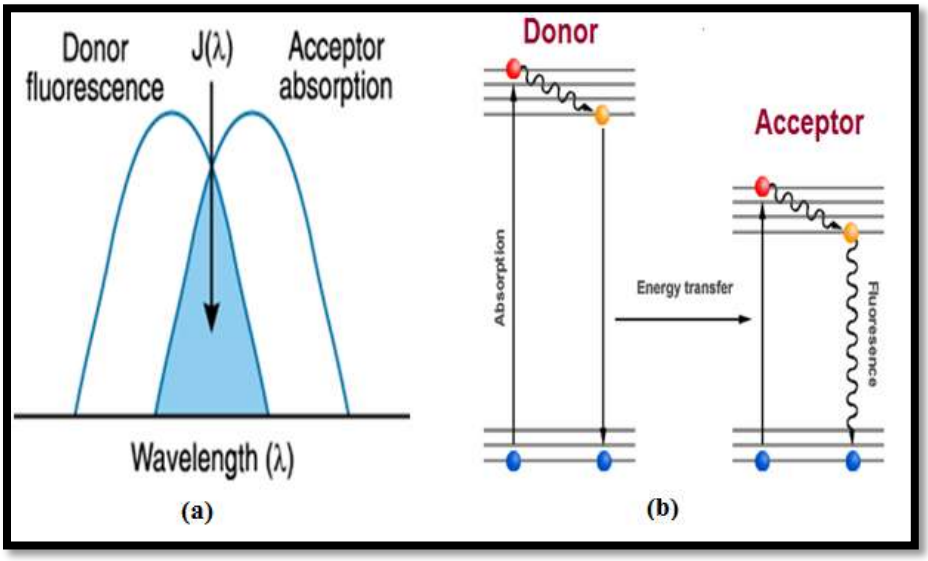

Figure 4: (a) Graph of FRET, (b) Jablonski diagram illustrating the FRET process.

Limitation of FRET:

i) Requirement for external illumination to initiate the fluorescence transfer.

ii) Can lead to direct excitation of the acceptor or to photo-bleaching.

To avoid this, Bioluminescence Resonance Energy Transfer (or **BRET**) is used, which uses a bioluminescent luciferase from sea pansy *Renillaren iformis*to produce an intialpoton.[43]

Fluorescence polarization (FP):

When fluorophore is irradiated with light, it gets exited and if remain steady throughout excitation state, it emits light in same polarized plane. While if it rotate and tumbles during excitation state, it emits light in different plane (depolarized). Larger molecule shows little movement while small molecule rotates quickly and gives high and low polarization value respectively FP is widely used in HTS.[44,45]

Applications:

i) This has been used to quantify biochemical properties such as protein denaturation, and attachment of proteins to nucleic acid etc.[46]

ii) Used to study receptor/ligand studies, Tyrosine Kinase Assays etc.

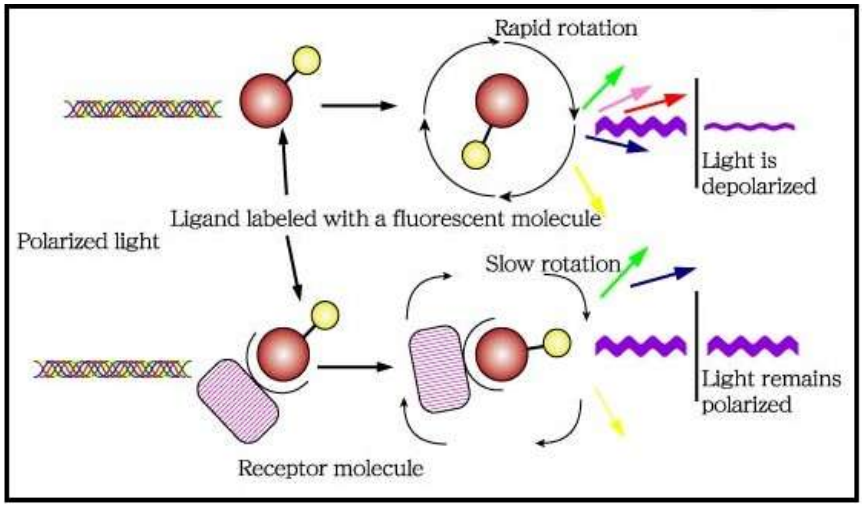

Figure 5: Fluorescence polarization.

Homogeneous time resolved fluorescence (HTRF):

HTRF (long lived fluorescence) consist of combination of standard FRET technology and time resolved measurement (TR) of fluorescence, allows elimination of short lived background fluorescence which occurs due to interfering material in the sample. It allows delay of approximately 50 to 150μseconds between the initial excitation and fluorescence measurement. HTRF uses the europium cry ptate (Eu^{3+}cryptate) as energy donor, which are rare earth complexes consisting of a macrocycle within which a Eu^{3+} ion is tightly embedded. This cage acts as antenna, collect and transfer energy to the Eu^{3+} ion, which eventuallyreleases this energy with a specific long lived fluorescent pattern.[47] Cryptate, have been used as fluorescent donor with cross-linked allophycocyanin or XL665, a phycobilli protein pigment purified from red algae as acceptor.[48]Liang et al. carried out a novel 384-well homogeneous time-resolved fluorescence leukotriene B_4assay for d the activity of leukotriene A_4hydrolase.[49]

It is a hybrid technique that takes advantage of the long fluorescence lifetimes of europium crytates and the large apparent Stokes shift (the difference between the peak excitation and peak emission wavelengths of a fluorophores) obtained by exploiting energy transfer between the europium donor and suitable acceptors. In an recent study HTRF as a screening application was used for the assay of tyrosine kinase and screening against tumor necrosis factor receptor in a 384-well microplate format.

Homogeneous time resolved fluorescence (HTRF)closely related to fluorescence intensity techniques. The detector is gated for a short period of time (e.g., 10 ns) -> the initial burst of fluorescence (most of the background fluorescence) not measured.

After the gating period the longer lasting fluorescence in the sample is measured. HTRF techniques can be used to substantially enhance sensitivity levels.

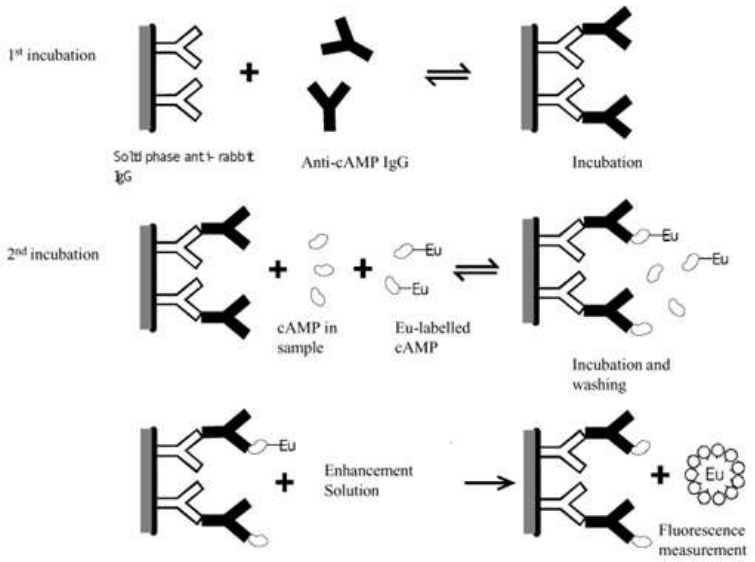

Figure 6: Homogeneous time resolved fluorescence (HTRF).

Applications of HTRF and FRET:

i) To study immunoassays and molecular interaction.[50]

ii) Various reagents pre-labelled with HTRF donors and acceptors, are existing and can be adapted to many assays.

Fluorescence correlation spectroscopy (FCS):

FCS is a confocal fluorescence technique in which parameter of prime importance is the fluctuation of the fluorescence intensity, occurs from noise as well as chemical, biological, and physical effects on the fluorophore. Different chemical and Physical changes, like equilibria, reactions, complexation, quenching and like molecular motion, photophysical interactions, and changes in conformation also affects the emission.

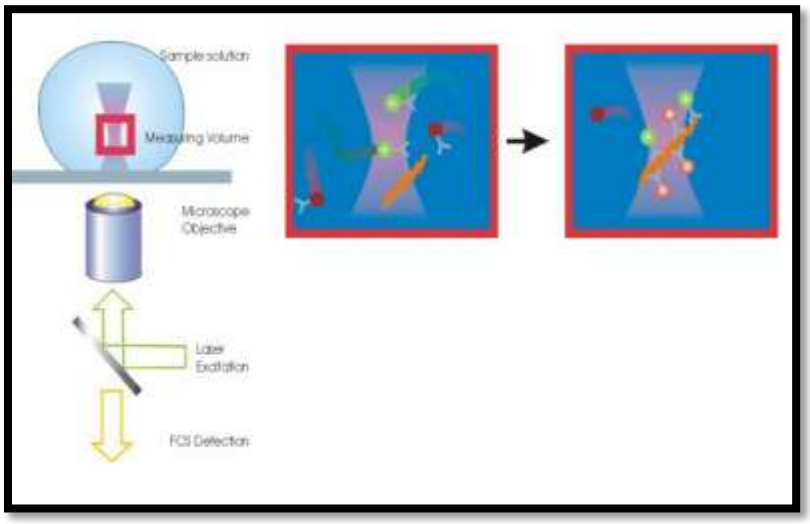

Figure 7: Fluorescence correlation spectroscopy(FCS).

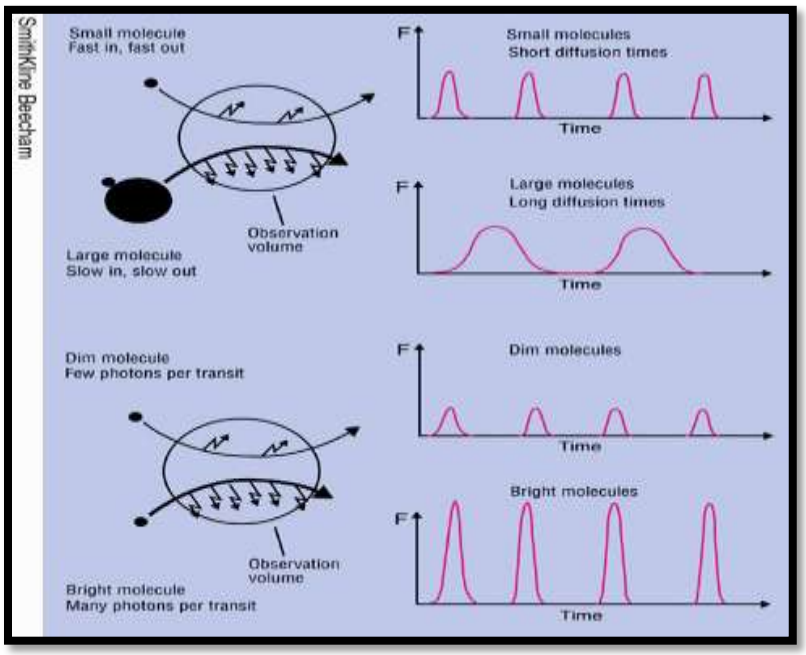

Figure 8: Graph of FCS.

Applications:

i) Determination of molecular interaction.

ii) Study conformation changes.

iii) Concentration and aggregation measurements, diffusion analysis.

iv) Can be used in binding assays and enzymatic assays.

v) Structural and molecular dynamics of fluorescent proteins in vivo and in vitro.[51,52]

Fluorescence intensity distribution analysis (FIDA):

FIDA is technique, developed for confocal microscopy which involves the monitoring of fluorescence intensity of a sample with a heterogeneous brightness profile. This method allows

the concurrent determination of concentrations and specific brightness values of a number of individual fluorescent species in solution.[53]

Nuclear magnetic resonance (NMR):

NMR based screening is a useful tool for lead identification, allows analysis of low molecular weight organic compound which bind to the protein targets, and gives affinity and binding location of potential lead compounds.[54] It is useful when hallow binding pockets need be targeted, when highly selective lead structures need to be designed etc. This technique is highly sensitive toward loose fragment binders and offers comparatively high throughput[55] It not only reveals the binding site or the conformation of the bound ligand but it can also provide information related with docking of the ligand to the protein's binding pocket[56]

2. Cell based assays[57]:

Cell-based assays for HTS can be classified under following classes:

Second messenger assays:

It monitors signal transduction from activated cell-surface receptors. Second messenger assays typically measure fast, transient fluorescent signals that occur in matter of seconds or milliseconds. Many fluorescent molecules are known to respond to changes in intracellular Calcium ion concentration, membrane potential and various other parameters, hence they are used in development of second messenger assays for receptor stimulation and ion-channel activation. The development of hydrophobic voltage-sensitive probes and FRET-compatible microplate instrumentation has helped the advancement of the screening technique for ion-channel drug discovery.

Reporter gene assays:

It monitors cellular responses at transcription/translation level. It indicates the presence or absence of a gene product that in turn reflects changes in a signal transduction pathway. The quantification of the reporter is usually carried out by biochemical methods viz by measuring the enzymatic activity. Plasmids are typical reporter genes employed. An entirely in vitro study was carried out by Suang Rungpragayphan et al. for generation and screening of combinatorial protein library in array format. This studied employed virtues of polymerase chain reaction (PCR) and in vitro coupled reporter gene assay.

Cell proliferation assays:

It monitors the overall growth/no growth responses of the cell to external stimuli. These are quick and easy to be employed for automation.

STATISTICS:

Selection of quality hits with high degree of confidence is important in drug discovery. When analytical method for hit selection is repeated under same or near about same conditions, result obtained differs with each other, and there is an obvious random source of variability in the system under study. The usage of statistical tools in the analysis of screening experiments is the right approach to the interpretation of screening data, which supports sound decision making. False positive results refers to the control assays that gives higher signal while, while false-negative refers to lower signal. Replicate measurements are preferred to guarantee the reproducibility of assay results.[58] The ability to identify true active compounds depends on the high quality of assays and proper analysis of data.[59] Z factor provides an easy means to assess the assay quality and it is used to examine the signal in particular assay.[60]

Z score, simple and wildly applied method for within plate normalization of all samples given by equation . 1

$$Z = \frac{x_i - \bar{x}}{s_x} \qquad \text{Eq. 1}$$

Where, xi is the raw measurement on the i^{th} compound, $\bar{x}$ and sx are the mean and the standard deviation, respectively, of all measurements within the plate.

B-score normalization procedure[58,61] is designed to remove plate row/column biases in HTS. The residual (rijp) of the measurement for row i and column j on the p-th plate is obtained by fitting a two-way median polish. In addition, for each plate p, the adjusted median absolute Deviation (MADp) is obtained from the r_{ijp}s. The B score is calculated by equation . 2

$$\text{B-Score} = \frac{r_{ijp}}{(1.4826 \times MAD_p)} \qquad \text{Eq. 2}$$

where, MAD_p = median

CONCLUSION:

HTS has importance in industry because of screening of large number of compounds daily. Various detection techniques FCS, NMR, HRTF etc, contribute to the screening of compounds in large number. HTS, usually to define a hit that is active on the target which can be converted to a genuine lead by chemical optimization, (with appropriate potency and selectivity) further pipelined in clinical development. Screening of excess of 100,000 samples per day (ultra-high throughput) has become possible because of utilization of high density plates, automation and miniaturization of assays which reduces time and cost, required for assays. HTS has a potential

to accelerate the drug discovery process.

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