FRET utility score to prioritise high-quality hit equity in High Throughput Screening

Delyan Ivanov[1], Antonia Turberville[1], Tiziana Monteverde[1]

[1]Hit Discovery, Discovery Sciences, R&D, AstraZeneca, Cambridge, UK

Introduction

Time-resolved Förster resonance energy transfer (TR-FRET) is one of the most common detection technologies (Figure 1) used in high-throughput screening to analyse molecular binding events or interactions for a wide range of target classes. Most commonly the ratio of acceptor to donor fluorescence is used to guard against fluorescence interference from compounds which equally affect both channels and interfere with the FRET signal. Although FRET artefacts are touted as relatively rare, in untargeted high-throughput screening campaigns where expected true hit rates are 0.1-0.5%, more than half of the actives in primary screens can be made up of artefacts. This means that many compounds are unnecessarily prioritised for downstream concentration-response testing in cascades, where they ultimately fail, wasting resources and slowing down hit discovery.

- We propose a utility score to rank actives from TR-FRET screens based on distance to the genuine behaviour of a known non-artefactual positive control.
- The score sums the ratio and a penalty term based on the sample point distance from the vector connecting the medians of untreated and positive controls.
- The score prioritises compounds which follow the spectral behaviour of genuine binders and can be tuned based on orthogonal assays or prior knowledge.

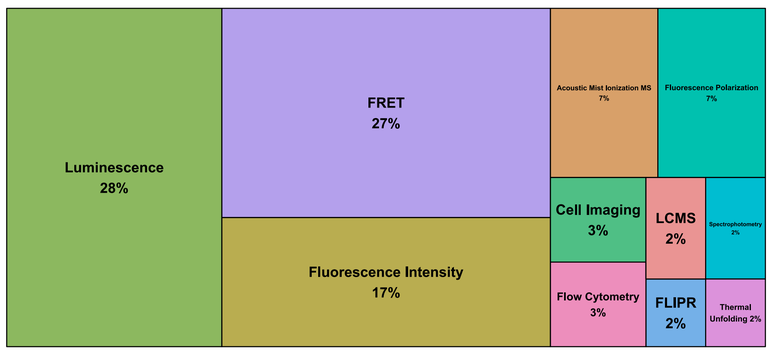


Figure 1: HTS Technologies applied in primary HTS campaigns in AstraZeneca over the past 10 years. FRET and luminescence are the two most common detection methods.

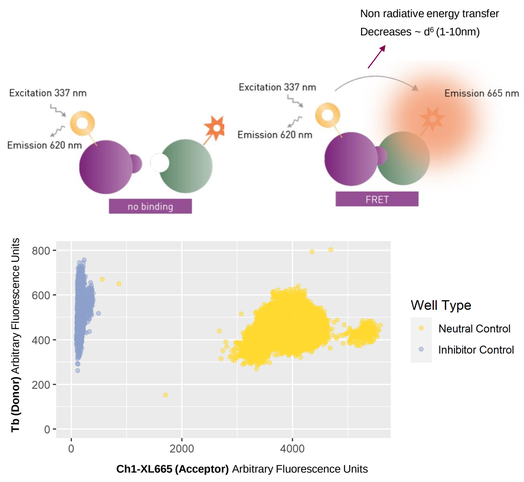


Figure 2: Principles of FRET (top) and exemplar behaviour (bottom) of untreated (neutral) controls and a positive (inhibitor) control.

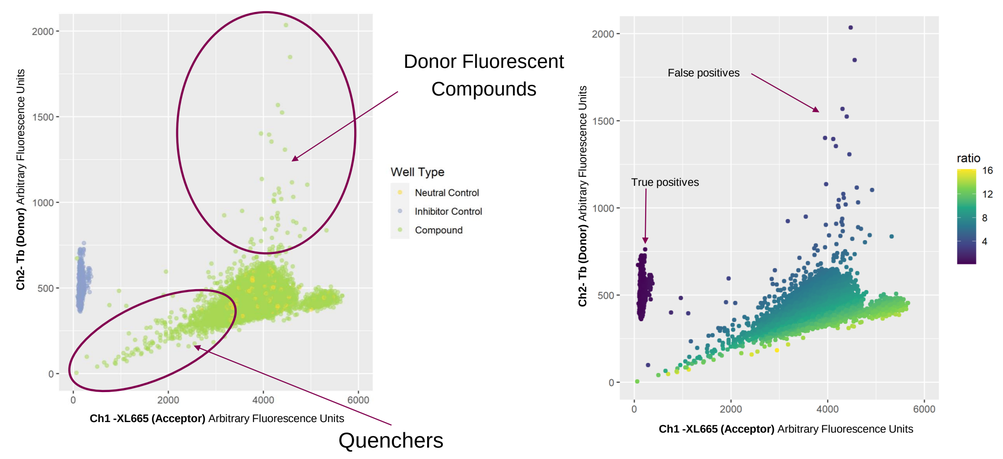


Figure 3: Spectral interference with FRET assays. Two main types of interference can be observed when plotting acceptor (Ch1) vs donor fluorescence (Ch2). Some compounds fluoresce in the donor channel, thereby showing lower apparent FRET ratios and no change in acceptor fluorescence. Other compounds act as inner filters and lower signal in both channels making the ratio unreliable.

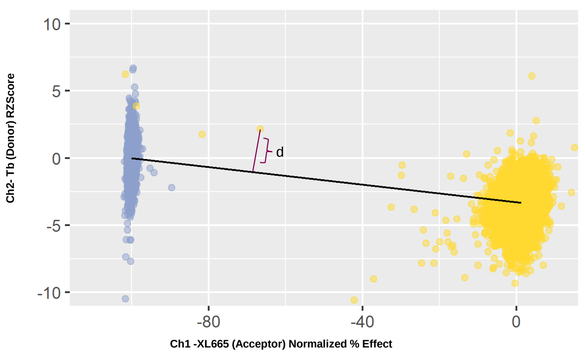


Figure 4. FRET penalty term "d" based on the sample point distance from the line connecting the median of the controls. Acceptor channel is normalised from -100 for the inhibitor (positive control) to 0 for the neutral (negative) control in yellow. Donor channel is normalised using the robust Zscore for the negative control. The units of the y-axis are median average deviations (MADs) from the median of the inhibitor control. Points further away from the line connecting the medians of the controls are considered less desirable.

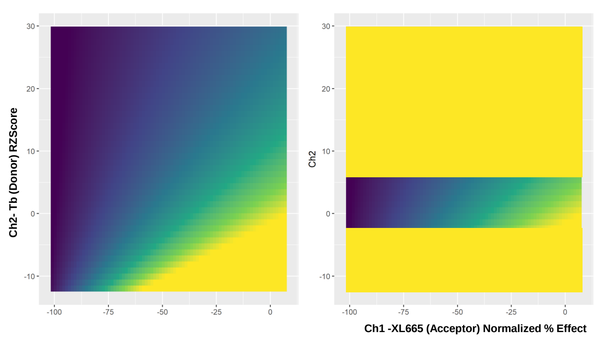


Figure 5. FRET utility in the normalised acceptor (Ch1) vs donor (Ch2) space Darker colours (blue) showing higher utility and lighter colours (yellow) indicating low utility. **A-** hit utility based on ratio penalises quenchers (yellow), but places high value (blue) on Donor fluorescence false positives; **B-** Removing artefacts using a sharp constant threshold in the donor channel that does not depend on magnitude of change in acceptor; **C-** proposed FRET Utility score penalises compounds which deviate from the vector of travel between the controls (yellow) with a tuneable n * d^2 term, placing high utility on compounds (blue), which follow the expected spectral behaviour. It allows for adaptive filtering and more leniency for compounds with stronger effects in the acceptor.

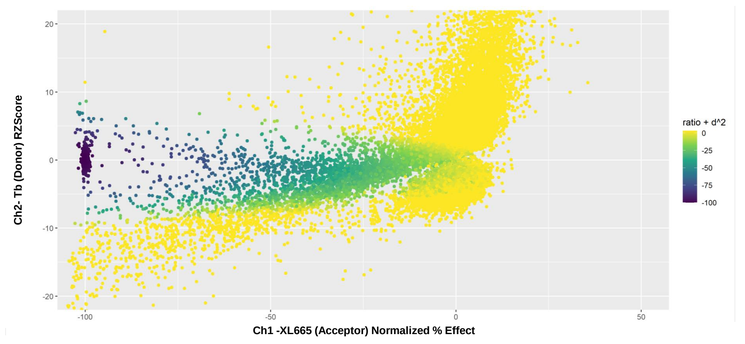
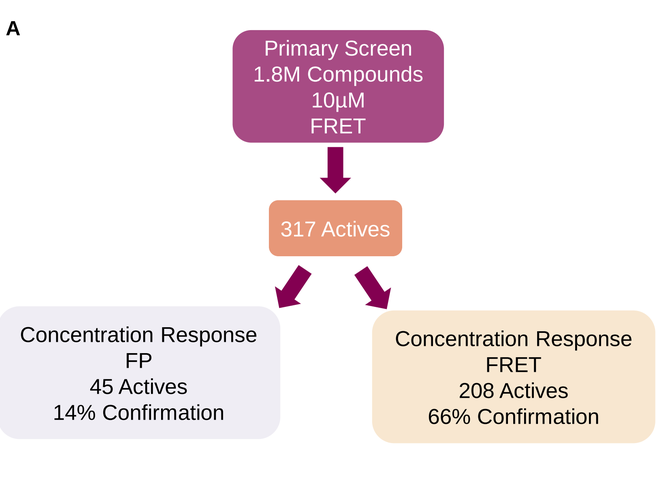
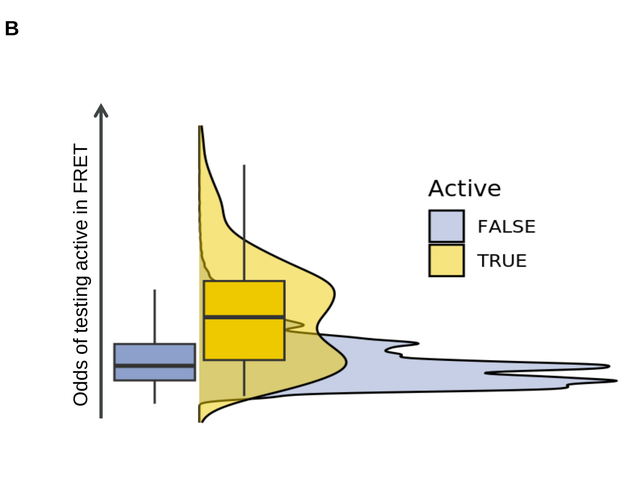
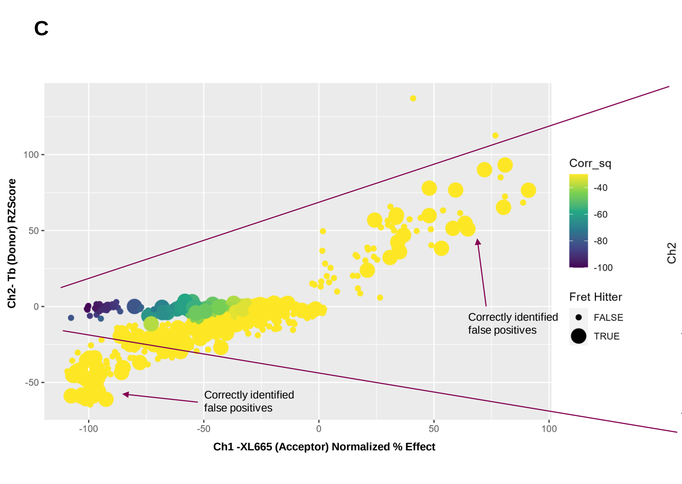


Figure 6. Example use of FRET utility in a primary HTS with high hit rate. Many of the actives in the FRET ratio show high fluorescence in the donor channel. The number of these compounds greatly exceeds the available capacity for concentration-response follow-up. However, using the FRET utility score, actives can be prioritised based on % effect and how closely they follow the expected spectral behaviour of FRET disruption. Thus, the FRET utility score enables ranking of compounds where capacity to follow up is exceeded.







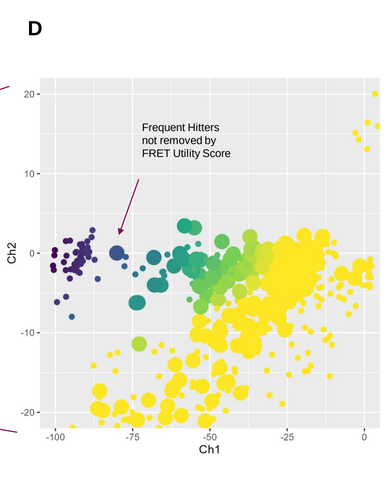


Figure 7. Use of FRET utility and HTS meta-analysis in removing artefacts and boosting confirmation rates. A- project cascade comprising of primary screen of a 1.8M compound library at a single 10 µM concentration; the primary screen actives are then followed up into concentration response assays in both FRET and fluorescence polarisation (FP). While confirmation in the FRET assay remains high (66%), only 14% of the primary actives confirm in the orthogonal technique assay pointing to a large proportion of artefacts. **B-** Profiling actives based on frequency of testing active in FRET Screens. Compared to the compounds in the collection testing inactive in the primary assay (blue), the primary actives (yellow) have a higher probability of testing active in other FRET assays (box plots). The population of primary actives shows bimodal distribution suggesting a mixture of false positive FRET artefacts and true positive genuine actives. **C-** Using FRET utility of actives to prioritise compounds to test in concentration response. Darker colours show higher utility, large dots indicate FRET frequent hitters defined as frequent actives in multiple FRET assays across many target types. FRET utility score removes a third of false positive frequent hitters based on quenching and donor fluorescence behaviour. **D-** Magnified view of C showing that some frequent hitters exhibit spectral behaviour consistent with FRET reduction reinforcing the use of historical HTS data to prioritise actives in conjunction with FRET utility score.

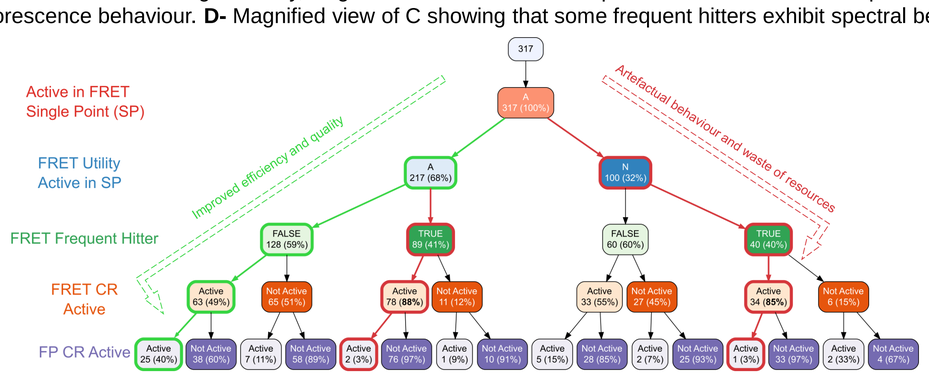


Figure 8. Breakdown of actives in the Primary screen split by hit utility active flag, classification as frequent hitter in FRET, and their behaviour in the FRET and FP concentration response (CR) screens. Compound prioritised by FRET utility score, which were not FRET artefacts and tested active in FRET concentration response, had **40% confirmation** in the orthogonal FP assay (**green path**). In contrast, compounds deprioritised by FRET utility and were labelled as FRET frequent hitters, had 85% confirmation in FRET CRs, but only **3% of them were active** in the FP assay **(red path).** This reinforces the utility of the orthogonal FP assay to remove artefacts. In cases, where orthogonal assay throughput is limiting or when time and resources need to be saved, using FRET utility and historical compound behaviour can substantially improve confirmation rates.

Conclusions

- Compounds prioritised based on FRET utility have higher confirmation rate in follow-up.
- FRET ratios can label artefacts as actives, wasting time and resources in hit identification cascades.
- Historical HTS meta-analysis removes FRET frequent hitters and further boosts confirmation rates.
- The >10-fold increase in downstream confirmation yields better quality hits at lower cost.
- The same approach can be applied for other ratiometric endpoints (e.g. FP).

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