Pattern Recognition Informed Feedback for Nanopore Detector Cheminformatics

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Abstract

Background

Pattern recognition-informed (PRI) feedback using channel current cheminformatics (CCC) software and LabWindows control software has been reported previously. The accuracy of the PRI classification was shown to inherit the high accuracy of the off-line classifier. For the molecular blockades studied here, the accuracy inherited is 99.9% to distinguish between terminal base pairs of two DNA hairpins. The pattern recognition software consists of Hidden Markov Model (HMM) feature extraction software, and Support Vector Machine (SVM) classification/clustering software that is optimized for data acquired on a nanopore channel detection system.

Results

The PRI-feedback system uses a server running a LabWindows protocol that is set up to control the amplifier voltage control settings to eject a molecule from the channel if not the desired class. The control server begins by taking signal information and broadcasting it via TCP/IP to a cluster of Linux computers to do the HMM and SVM computations, and decision-making, in a distributed setting. For general nanopore detection, the distributed HMM and SVM processing provides a processing speed-up that allows the pattern recognition process to complete within the time-frame of the signal acquisition in-process – where the sampling is halted if the blockade signal is identified as not in the desired subset of events (or once recognized as non-diagnostic in general). Successful operation of the above system is shown in numerous settings.

Conclusions

Due to purification limitations Nanopore Detection with PRI offers significant advantage when seeking data acquisition for an antibody-antigen system due to the reduction in wasted observation time on eventually rejected “junk” (non-diagnostic) signals. The use of PRI feedback for nanopore channel data collection greatly improves data acquisition capabilities overall and may eventually provide a means for nanomanipulation of single biomolecules.

Introduction

Pattern recognition informed sampling and experimental feedback opens the door to a whole new realm of signal stabilization and device capabilities. In this paper we describe results from recent pattern recognition informed (PRI) sampling experiments on the nanopore detector platform [1-3]. The signal analysis involves use of Machine Learning algorithms such as hidden Markov models (HMMs) [2,4-6] and Support Vector Machines (SVMs) [7-10]. The Machine Learning (ML) algorithms are amenable to distributed computational solutions (for the HMMs, in particular [11]), which permits a computational speedup that ensures real-time operational feedback on the nanopore detector applications studied. Although a specific application is examined here in detail, a similar approach can be used in many experimental efforts. The results and application of the PRI method, with distributed HMM and SVM algorithms are generally applicable in knowledge discovery contexts involving stochastic sequential analysis and/or classification/clustering. Some background material on the Nanopore Detector and the Channel Current Cheminformatics Architecture are given in what follows.

Nanopore Background

A nanometer-scale channel can be used to associate ionic current measurements with single-molecule channel blockades. The alpha-Hemolysin channel, used in the experiments described here, self-assembles such that a single channel of alpha-Hemolysin can be isolated in lipid bilayer. This provides an inexpensive and highly reproducible, method to construct a nanopore-based detector that is informed by single molecule interactions with a nanometer scale channel. See [1,2] for further details. An example of a direct interaction/modulation of the channel ion current is shown in [3]. Indirect channel modulation, or transduction, is described in [12], and is being used for generalized binding analysis at the single interaction-complex level.

The pattern recognition informed (PRI) sampling is done with mixtures involving the five DNA hairpin control molecules examined in [1,2] (see Methods for sequence details). These hairpin molecules have stem lengths with only eight or nine base-pairs, are blunt ended on one end, and have a hairpin loop consisting of 4 dT's. When lengthy blockades are observed at the nanopore detector, the associated molecules are hypothesized to be captured at their blunt ends. Early tests, with dumb-bell shaped molecules involving 4 dT loops at both ends, showed no lengthy blockades, consistent with this hypothesis [1]. When captured at their blunt ends the molecules are hypothesized to be held at their loop ends near the alpha-Hemolysin channel opening by steric hindrance (with one loop state in standard conditions, see Methods). The stem length is selected to be sufficiently long to have transient binding interactions with the high charge channel residues near the limiting aperture [13], but not so long that any one bound state or single steric constraint dominates. This optimization/selection on control molecules, as regards stem length tuning or base-pair alterations, is then redone as necessary when these same control molecules are covalently linked to sensing moieties of interest (see nanopore transduction detection [12,14]).

Channel Current Cheminformatics Architecture

In [6] we describe preliminary work to establish real-time control of a nanopore detector. The work is based on live, streaming, measurements and fast pattern recognition identification of blockading ("captured") analytes. Real-time *sampling* control of a nanopore detector, alone, has been proposed to boost nanopore detector sampling productivity by orders of magnitude, depending on the mix of desirable signal classes vs. undesirable in the data being analyzed. In a real-time setting the challenge is to perform the feature extraction sufficiently quickly (whereas the SVM is trained off-line, so operates very quickly on-line). In this work we show that this can be accomplished with fast, distributed, HMM processes.

The real-time experimental linkage between the DAQ and the computational facilities is implemented using a LabWindows experimental-feedback coding environment that connects via TCP/IP to a cluster of Machine Learning nodes that run our "in-house" Channel Current Cheminformatics (CCC) methods (see Methods). Data acquired with LabWindows is passed to the network of CCC software clients on a streaming real time basis. The classification results are then quickly returned to the LabWindows automation software for experimental feedback control. As suggested in [6], the real-time classification inherits the 99.9% accuracy of the non real-time implementation (established in prior work [2]) as nothing has changed in regards to the features extracted and the classifier used. Thus the full power of HMM and SVM methodologies can be leveraged into numerous “real-time” experimental protocols that would employ PRI methods.

Methods and discussion

Nanopore Detector

The experimental setup is described in detail in [1,2]. Each experiment is conducted using one α-hemolysin channel inserted into a diphytanoyl-phosphatidylcholine/hexadecane bilayer across a 25-micron-diameter horizontal Teflon aperture, as described previously [1,2]. Seventy microliter chambers on either side of the bilayer contains 1.0 M KCl buffered at pH 8.0 (10 mM HEPES/KOH) except in the case of buffer experiments where the salt concentration, pH, or identity may be varied. Voltage is applied across the bilayer between Ag-AgCl electrodes. DNA control probes are added to the *cis* chamber at 10 or 20 μM final concentration. All experiments are maintained at room temperature (23 ± 0.1 $^{\circ}$C), using a Peltier device.

Test Molecules

The eight base-pair and nine base-pair hairpin molecules used in this study were previously studied in [1,2,13]. The full sequence for the 9CG hairpin is 5' GCGCGCGCGTTTTTCGCGCGCGC 3', where the base-pairing region is underlined. The eight base-pair DNA hairpin is identical to the core nine base-pair subsequence, except the terminal base-pair is 5'-G•C-3'. In the middle of the loop, a dT residue was conjugated with biotin (through a six carbon linker). The prediction that each hairpin would adopt one base-paired structure was tested and confirmed using the DNA mfold server [15].

Channel Current Cheminformatics

A capture signal generated with the nanopore apparatus is filtered and amplified before it is sent through the DAQ. The data acquisition device converts the analog signal to digital format for use in the display and recording of data in binary Axon (Molecular Devices) format. In the pattern recognition feedback loop, the first 200 ms detected after drop from baseline are sent via TCP-IP protocol to the HMM software, which generates a profile for each signal sent. The HMM-generated profile is processed with the SVM classifier to compare the real-time signal with previous training data in order to determine whether the signal is acceptable (see Fig. 1). If the signal is acceptable, the message to continue recording is sent to the LabWindows software to continue recording, and the molecule is not ejected from the channel by the amplifier. If not, a message is sent to LabWindows to eject the molecule, and the amplifier briefly reverses the polarity to eject the molecule from the channel (see Fig. 2).

For the successful real-time feedback experiments described in the Results, only two computers, a client and a server were needed. In general, the server consist of a cluster of computers to distribute the HMM, and possibly SVM, processes. The Client runs Microsoft Windows XP to visualize and record the entire experiment by using LabWindows. Our in-house implementation of LabWindows acquisition software is able to detect blockades using a tFSA, while also recording and visualizing the experiment. Our implementation for channel current analysis also has the critical functionality to change the polarity of current, so as to eject any molecules pore when necessary. The Server computer runs Pardus Linux 2007.3. The hardware for both the Client and Server consists of PCs with 2.4GHz AMD CPUs, with 2GB memory.

Client side signal processing

1. Run LabWindows to start visualizing and recording the signals from the DAQ, which is connected to the experiment environment (see Fig. 2).

2. Connect and ask Feedback Server for the existing SVM models, select the appropriate SVM model according to the type of the molecules in the experiment and select the desired molecule class (e.g. if we have an SVM model for 9GC/9TA, we may keep 9GC molecules or 9TA's). In the Results in one experiment we used the "9GC/9TA" model in the "Keep 9GC" PRI mode. Then we also examine the "6GC/7CG/8GC/9GC/9TA" model in "Keep 9GC" PRI mode.

3. Acquire data from the experiment environment at 50KHz sample rate.

4. Perform tFSA on every 1 second worth of data to find out if there is any molecular blockade.

5. In case of a blockade, connect to Feedback Server over TCP/IP and send 300ms worth of sample data with the desired SVM classification model.

6. According to servers response take action: if server advises rejection of the molecule - and if that molecule still in the nanopore - eject the molecule from the nanopore by

changing the polarity in three steps (change polarity negatively, go to 0, go back to normal current). Go back to 3 and loop this process until the experiment is halted.

Server side signal processing

1. Handle incoming connections

* There are couple of possible requests from server according to our communication protocol, a client might be asking for SVM models, might be asking to classify a sample data according to any particular SVM model.

* The Server handles the overwritten requests; molecule might leave the nanopore before feedback servers response and if client sends another request in the middle of another.

* The Server is multi-threaded and can have hundreds of SVM models for different type of experiments. Client can query server for different models, dynamically. This also enables server to handle multiple clients with different needs for different type of experiments seamlessly. Response time is around 1.8 seconds in our local network with a one-computer (test) cluster for the Client, where a speedup of approx 100 for a 25 quad-nodes (each running 4 threads of computation) is possible without difficulty.

2. If there is a feedback request, start a thread to read the sample data from socket and stream it to the HMM processing to extract features.

3. Feed SVM with the resulting HMM feature vector from (2) and apply the desired SVM model for classification.

4. Compare the predicted class with the expected class of clients request. If classes match, return KEEP, otherwise return REJECT feedback to the client.

Distributed HMM feature extraction

The HMM implements 50 states as determined by making 50 bins of the blockade current data. The quantized data goes through one round of Expectation-Maximization to obtain transition probabilities after running the Viterbi algorithm to obtain the most probable path of states that created the signal (more de-noising). The 150-component feature vectors are determined for the signal, consisting of three groups of 50: the first 50 features comprise the Viterbi path level occupations, the second 50 are the emission variances, and the last 50 are a compression of the level transition information (see [2] for details).

Distributed SVM classification

Support Vector Machines are a variational calculus based method constrained with structural risk minimization. The SVM determines a hyperplane to optimally separate one class from another, as determined by training. The kernel choice provides a “distance” from points to the hyperplane, which defines separation. The choice of kernel determines the performance in classification. The distance of points from the hyperplane, determine the confidence levels in how well sample data has been classified. A confidence level

greater than 60% is used here as the cut-off to determine whether to accept or reject a live signal.

Currently there are two approaches to implementing *multiclass* SVMs. One arranges several *binary* classifiers as a decision tree such that they perform a multi-class decision-making function (SVM-external classification – this is the classifier architecture used here, see [2] for further details). The second approach involves solving a single optimization problem corresponding to the entire data set (with multiple hyperplanes), with multi-class discriminator optimization performed internally. The SVM-internal approach, when it is stable and properly generalizable (an active area of research), is preferred, since a tuning over Decision tree topologies and weightings is avoided [9]. The on-line discriminatory speed of a trained SVM is simply that of evaluating an inner product, so it's operational constraint on the PRI feedback endeavor is negligible compared to that of the HMM feature extraction stage. For this reason, there is little discussion of SVMs in this paper, even though SVMs comprise much of the complexity of the HMM/SVM PRI feedback system.

Results

Binary PRI Sampling

The nanopore experiments with PRI sampling are first done with a 1:70 mixture of 9GC:9TA. Figures 3 and 4 show how molecules appear in terms of their blockade attributes in the on-line setting (with event-observation time on the vertical axis in Fig. 4). In Figure 5 the PRI sampling acquisition results are shown, with the rarer 9GC molecules properly identified, and sampled for a full 5 second duration, while others molecules are rejected, typically in a fraction of a second (with the prototype network setup used here).

Multi-class PRI Sampling

The robustness of the results are then explored when there are numerous other classes present (see Fig. 6). In Figures 7 and 8 an approximately 1:70 mixture of 9GC:{6GC,7GC,8GC,9TA} is examined, with 9GC sample time again boosted correctly as indicated.

State resolution during binding analysis

The applicability of the nanopore transduction method to binding analysis is then tested by examining a biotinylated DNA hairpin and its interactions with streptavidin (see [12] for complete details). The biotinylated DNA hairpin (Bt-8GC) is constructed from the 8GC control molecule, where a modified thymine is introduced at the midpoint of the 4 dT loop. The modified thymine has a 6 carbon spacer arm that connects to a sterically unhindered biotin molecule.

In [12] it is shown how bound and unbound states resolve via use of a transduction molecule (here the biotinylated 8GC DNA hairpin). It is found, however, that the bound-state has two blockade states. These two states are not thought to be due to two binding conformations, but are hypothesized to be due to two conformations on the hairpin loop. The hypothesis of two loop states is tested in Figure 9, where it is shown that two DNA hairpin states can be induced by introduction of sufficient chaotropic agent (3.5 M Urea).

Discussion

Sample Boosting and Nanomanipulation via PRI Selection

PRI sampling is done with mixtures involving the five DNA hairpin control molecules examined in [1,2,13]. In the nanopore transduction detection context [3,14], it is hypothesized that auxiliary molecules consisting of these same control molecules can be covalently linked to sensing moieties of interest to provide the beginnings of a generalized detection platform. Once covalently linked, however, further optimization/selection on the control molecule portion, as regards stem length tuning or base-pair alterations, is usually needed to reacquire a highly structured modulatory signal (with stationary statistics) [12]. Once a bifunctional molecule has been engineered to desired channel modulation and target-analyte interaction, the nanopore detector can be operated with the transduction molecule and signal analysis software to classify the different blockade signals. As far as the signal processing software is concerned, however, pattern recognition that resolves different hairpin blockades, or the same hairpin blockader with/without complexation at its binding moiety, is practically the same. Thus, the five DNA hairpin PRI-sampling study examined here demonstrates a capability for nanomanipulation when observing reactants, via the mechanism of recognition and appropriate selection.

PRI Digital Stabilization

The ramifications of real-time pattern recognition on a digitized stream of experimental output (or any device output) are profound. One capability is introduction of a carrier reference (CR) molecules for device stabilization. The CR bifunctional molecules are selected/designed for their sensitivity to different buffer parameters, such as pH or salt content. These nanopore transduction molecules are included during nanopore detection of the analytes of interest. With PRI feedback, any pH or concentration drift indicated by the CR's can be countered by appropriate microfluidics controls, to provide an exceptionally stable experimental environment. Without PRI, corrected signal, analogous to the signal seem when the buffer is held fixed experimentally, can be reconstructed using changes indicated by the CR signals (embedded in the data stream). This can only work if buffer variations are kept very small, however, so for practical usage the PRI capability is essential.

Strong pattern recognition capabilities with the classes to be discerned also affords the opportunity to directly encode the CR indication of instrument state in an associative memory context with the observed (non-control) blockade signal. This is simply done by altering the non-control feature vector to be itself concatenated with the last seen control-signal feature vector.

Exploring the Ergodic Hypothesis at the single-molecule level

The nanopore system gives us a single molecule view of individual molecular interactions. The lifetimes of sub-blockade levels reveal information about the interaction kinetics of the captured molecule -- resulting from interactions with another molecule (the binding studies), from interactions with the channel, or from (internal) conformational changes. We get precise kinetic data, in other words, from careful

observation of sub-blockade lifetimes. Inherent to this hypothesis is application of the classic ergodic hypothesis (roughly speaking, that ensemble statistical averages equal time-averages of individual histories). Thus, as single molecule studies are carried out, a new level of exploration of the ergodic hypothesis will be inherent to that effort.

Conclusions

The primary purpose of this experiment was to develop an implementation of the pattern recognition informed (PRI) experimental protocol for more specific and efficient collection of signals in nanopore cheminformatics experiments. In the Results, PRI-sampling is shown to boost the acquisition rates on molecules of interest by orders of magnitude, greatly extending the applicability of the Nanopore's inherent serial-event detection capability.

A secondary purpose was to explore the resolving/tracking power of the PRI system when applied to binding experiments. The clear binding behavior shown ("tracked") in the Results indicates that population-based binding studies using the nanopore detector can be done, and suggests that sufficient sensitivity to state might be possible for tracking an individual binding history in future efforts along these lines.

Competing interests

The author declares that there are no competing interests.

Author's Contribution

AME did the computational side of the PRI experiments. The nanopore detector experiments were setup and operated by IA, AA, and EM. Gel validation tests were performed by AS. The cheminformatics signal analysis was done using software developed by SWH. The paper was written by SWH and read and edited by the coauthors.

Acknowledgments

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Figure Legends

Figure 1. Labwindows/Feedback Server Architecture with Distributed CCC processing. The HMM learning (on-line) and SVM learning (off-line), denoted in orange, are network distributed processes for N-fold speed-up, where N is the number of computational threads in your cluster network.

Figure 2. Operational view of Nanopore Detector sampling control.

Figure 3. Standard deviation vs. Mean on a {9GC,9TA} mixture in a 1:70 ratio. Clusters of 9GC and 9TA signal groups identifiable, nearly full blockade signal group also present.

Figure 4. Standard deviation vs. Mean vs event-observation time (vertical axis). Drift in the {9GC,9TA} signal is seen as the experiment proceeds due to evaporative

concentration of the background salt. This results in altered environment for the DNA hairpins, one where the increasing magnitude of the blockade std. dev.s is thought to be due to stronger (and noisier) DNA hairpin channel blockades.

Figure 5. Standard deviation vs. Mean vs event-observation time vs PRI-informed sample observation time (4th dimension represented as the radius of the data point). This figure shows a successful real-time operation on the PRI-sampling method on the ND platform. 9GC signal is selected for observation and it is at a 1:70 lower concentration than the decoy 9TA DNA hairpins. As can be seen, only 9GC signals are held for the lengthier observation time, all other molecules being rejected promptly upon identification (the smaller diameter events points correspond to short lived events), where the brief duration of the event is dictated by the active, PRI-control, of the device voltage.

Figure 6. Standard deviation vs. Mean on a {6GC,7GC,8GC,9GC,9TA} mixture. Clusters of the different species of blockade signal are clearly identifiable (and the nearly full blockade signal class is also present).

Figure 7. Standard deviation vs. Mean vs event-observation time (vertical axis) vs PRI-informed sample observation time (4th dimension represented as the radius of the data point). Drift in the signal is seen as the experiment proceeds, as before. Similar strong classification performance is demonstrated for this five-class test as with the prior two-class test.

Figure 8. Shows a rotated view of the results shown in Fig. 7. The successful 99.9% accurate separation of the 9GC from the {6GC,7GC,8GC,9TA} signals can be seen more clearly from this perspective. Note: the actual discriminating features used by the SVM classifier are not based on the mean and standard deviation statistical features plotted, but on a 150-component feature extraction based on HMM emission and transition probabilities, and Viterbi-path statistics (see [2] for further details).

Figure 9. Sufficiently strong Urea concentration (5 M) again results in racemization of the two loop capture-variants, while weaker urea (notably at 2 M) does not. The results shown here are consistent with the two-state loop hypothesis, and suggest that the observation of such (see [12]) is NOT due to weak urea content (since it is present not at all, or in 2 M concentration, in that experiment).

Figures

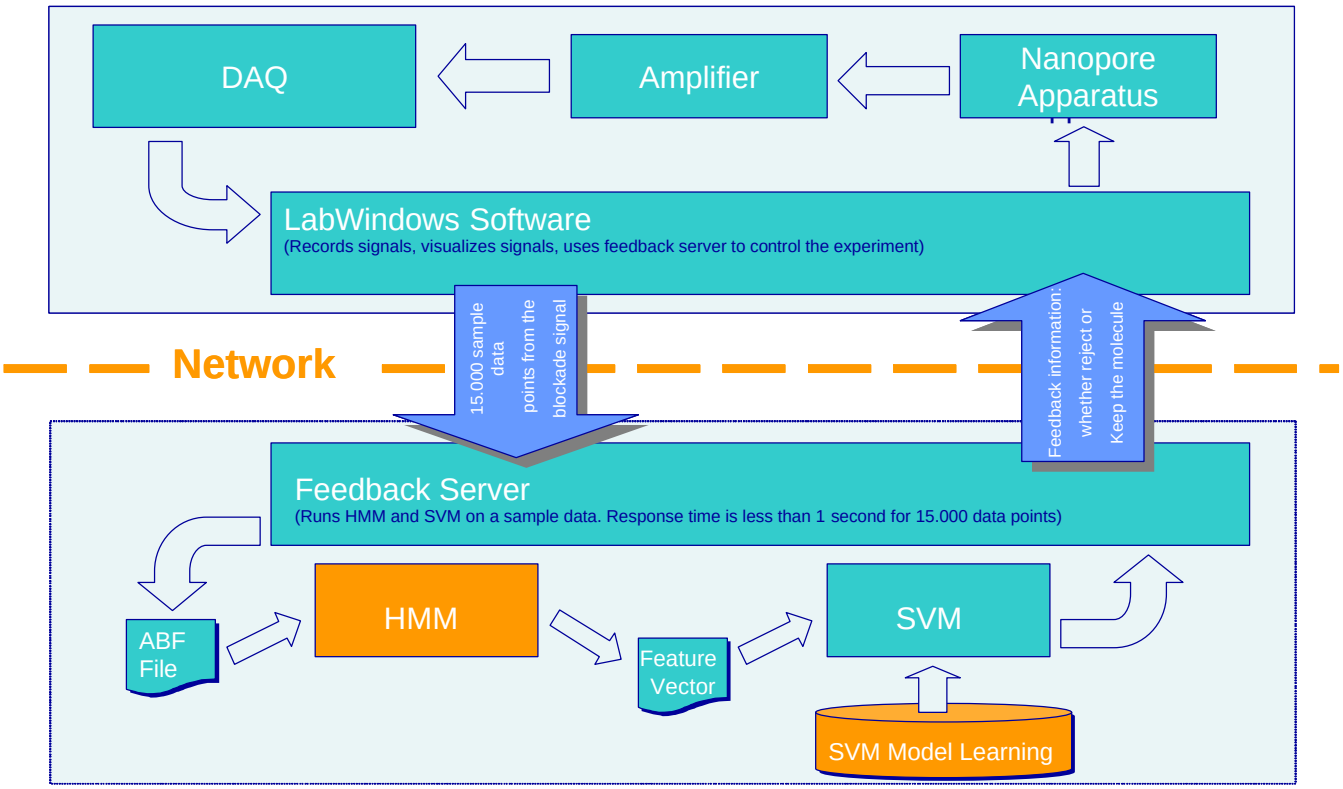
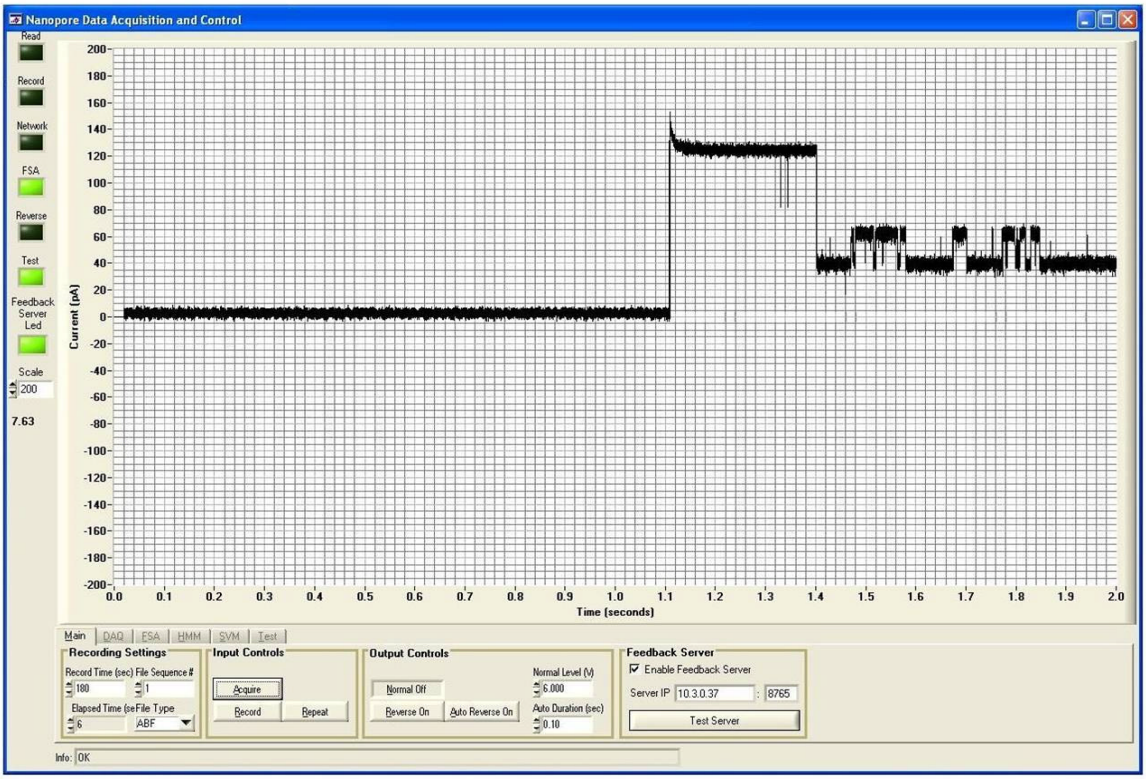


Figure 1.

Run client, enable Feedback server support and start experiment:



Once the molecule is captured, client sends a sample portion of data to the server to get the feedback information about the molecule:

```
meren@pardus-cerm Feedback Server $ python server.py 8765
* Server is up and running at '10.3.0.37:8765'

Incoming Data
.....................................................................................................................................................
.....................................................................................................................................................
.

* Calling request handler...
Request Type     : SIG, TID:  1201560206.7
Signal count     : 15012
Signals          : 39.3676739114, 38.4521466111, 38.7573223779, 42.4194315789  ...  39.3676739114, 41.1987285119, 39.3676739114, 39.6728496781
Average Baseline : 125.022111331

* Creating an ABF file with sample signals...
ABF file         : '/home/meren/repos/winters-lab/trunk/LabWindows/Feedback Server/working_space/1201560206.7/test.abf'

* Running HMM on ABF file to extract feature vector...
Feature vector   : '/home/meren/repos/winters-lab/trunk/LabWindows/Feedback Server/working_space/1201560206.7/feature_vector'
Property file    : '/home/meren/repos/winters-lab/trunk/LabWindows/Feedback Server/working_space/1201560206.7/SVMTestInput.properties'

* Running SVM on the feature vector...
Predicted Class : -1.0
Result:  OK
Total data sent:  3
```

Figure 2.

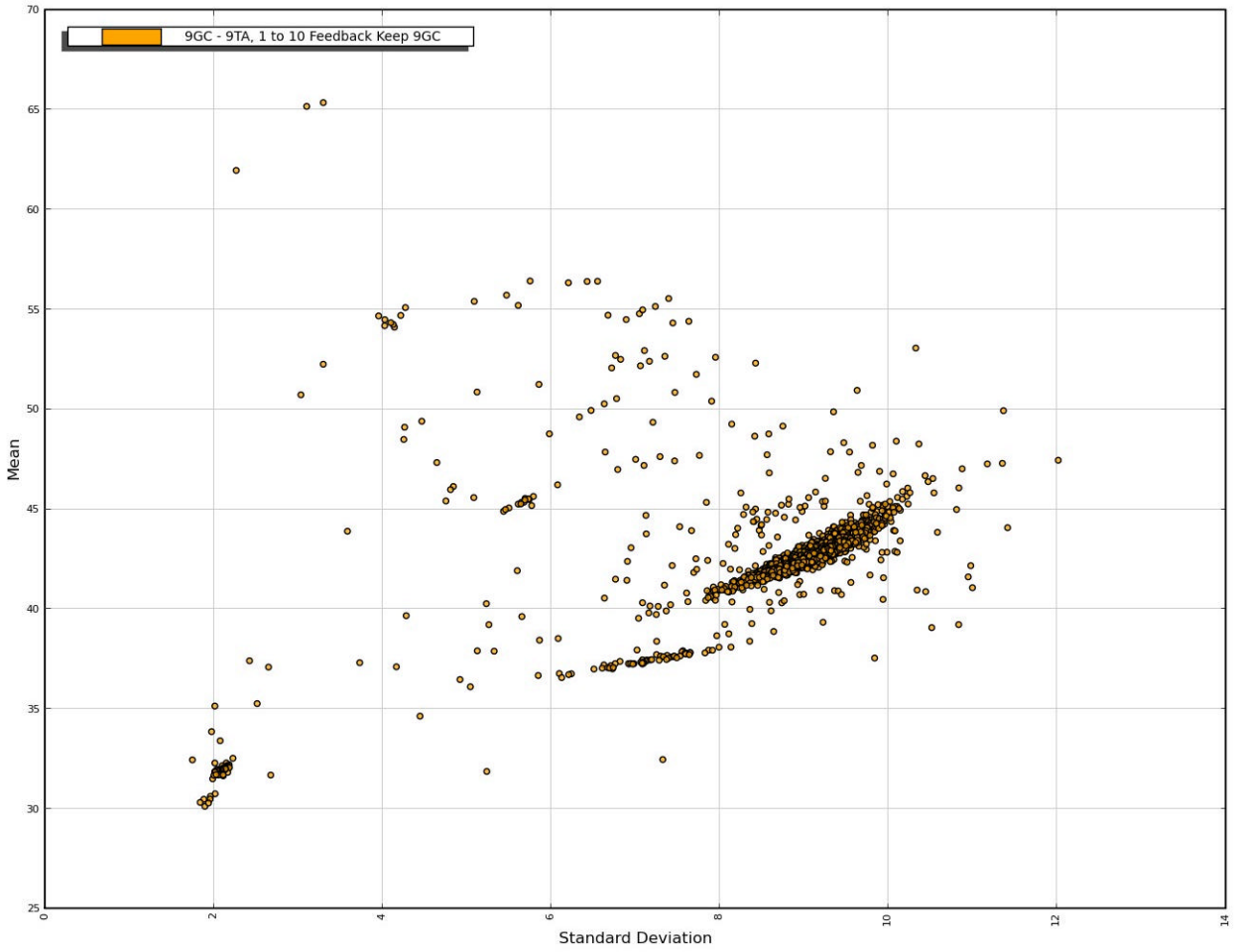

9GC - 9TA, 1 to 10 Feedback Keep 9GC
Mean
Standard Deviation
70
65
60
55
50
45
40
35
30
25
0
2
4
6
8
10
12
14

Figure 3.

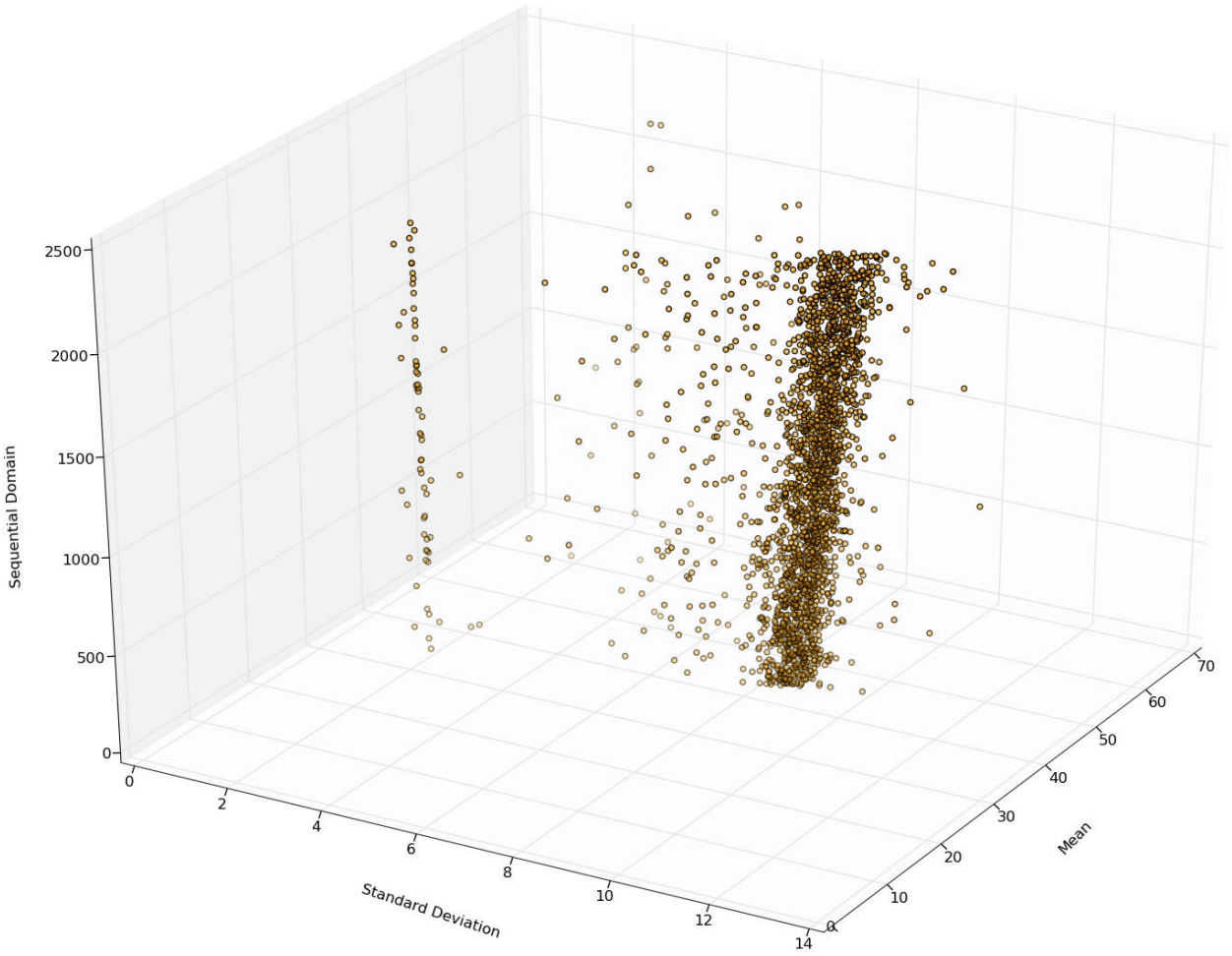

Sequential Domain
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
Standard Deviation
0
10
20
30
40
50
60
70
Mean

Fig. 4

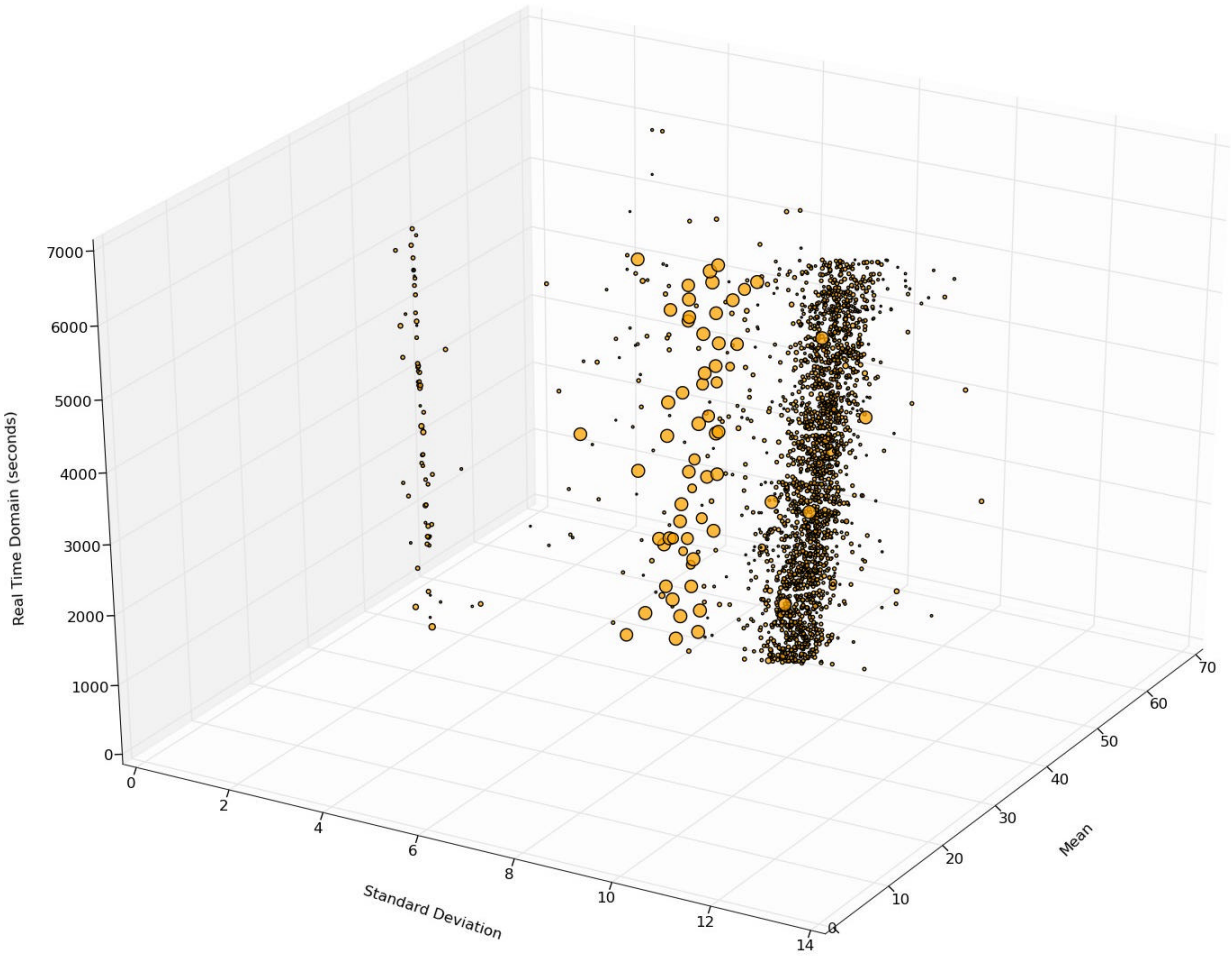

Real Time Domain (seconds)
7000
6000
5000
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
Standard Deviation
0
10
20
30
40
50
60
70
Mean

Fig. 5

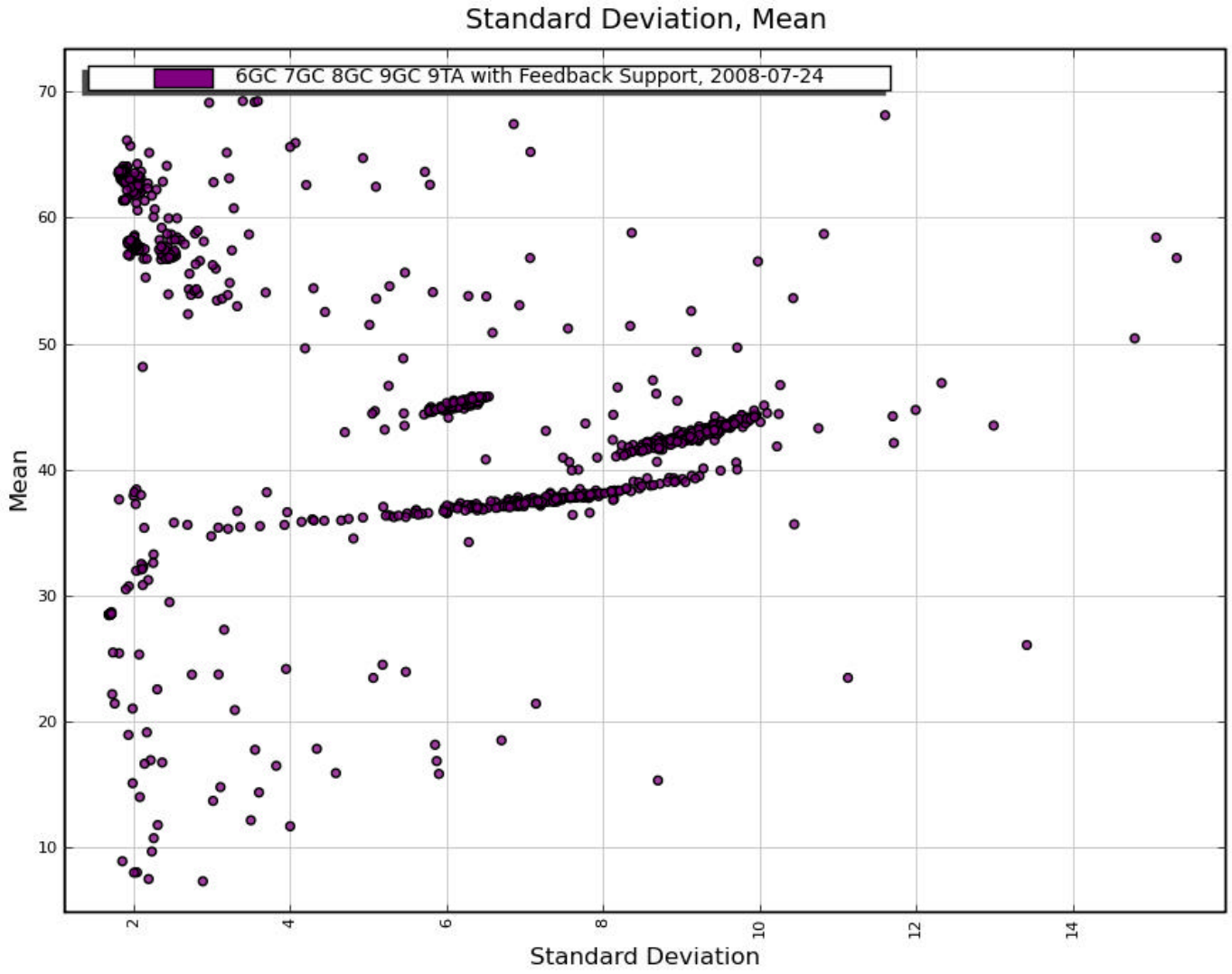

Standard Deviation, Mean
6GC 7GC 8GC 9GC 9TA with Feedback Support, 2008-07-24
Mean
Standard Deviation
70
60
50
40
30
20
10
2
4
6
8
10
12
14

Fig. 6

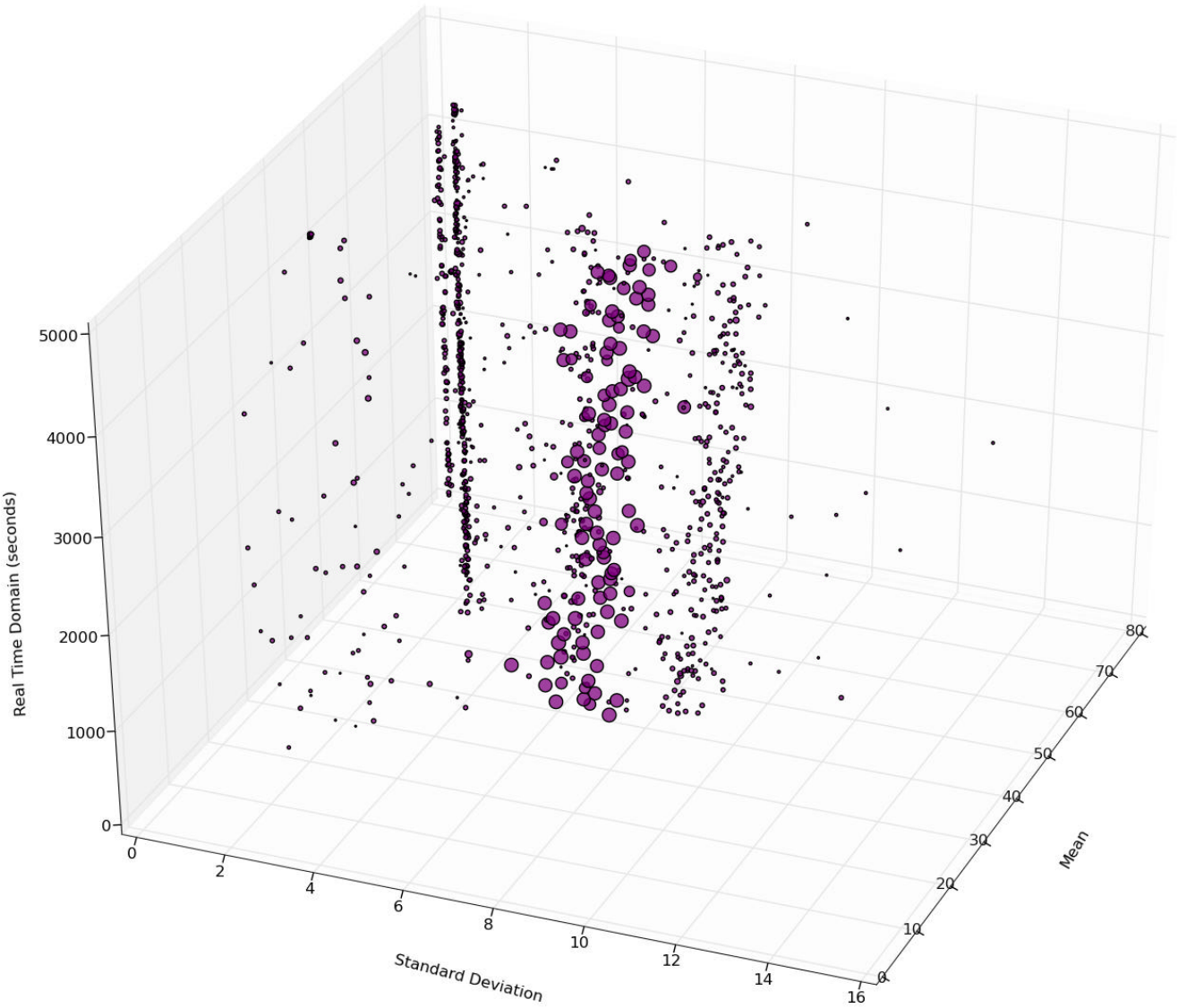

Real Time Domain (seconds)
5000
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
Standard Deviation
0
10
20
30
40
50
60
70
80
Mean

Fig. 7.

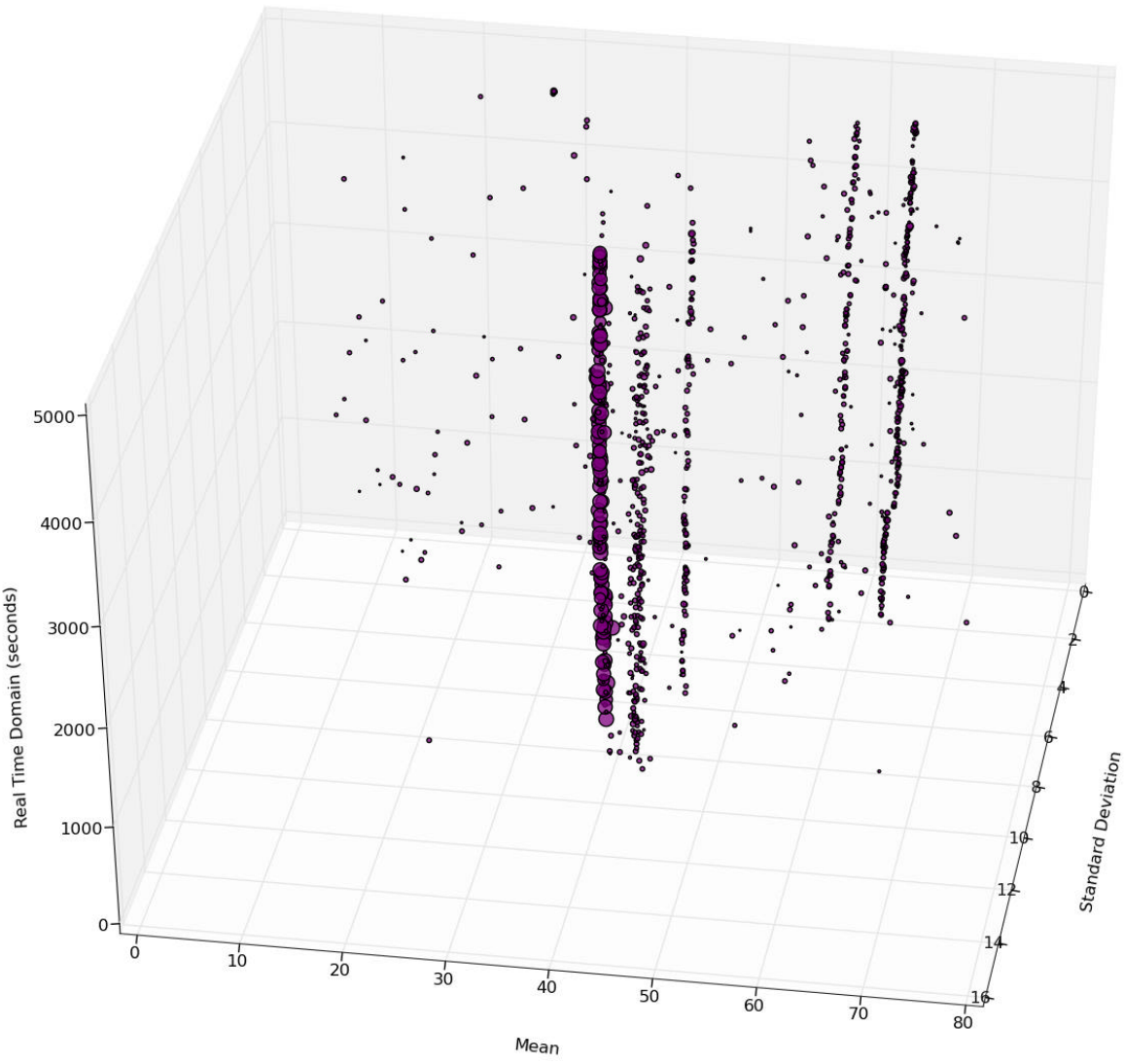

Real Time Domain (seconds)
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
Mean
0
2
4
6
8
10
12
14
16
Standard Deviation

Fig. 8.

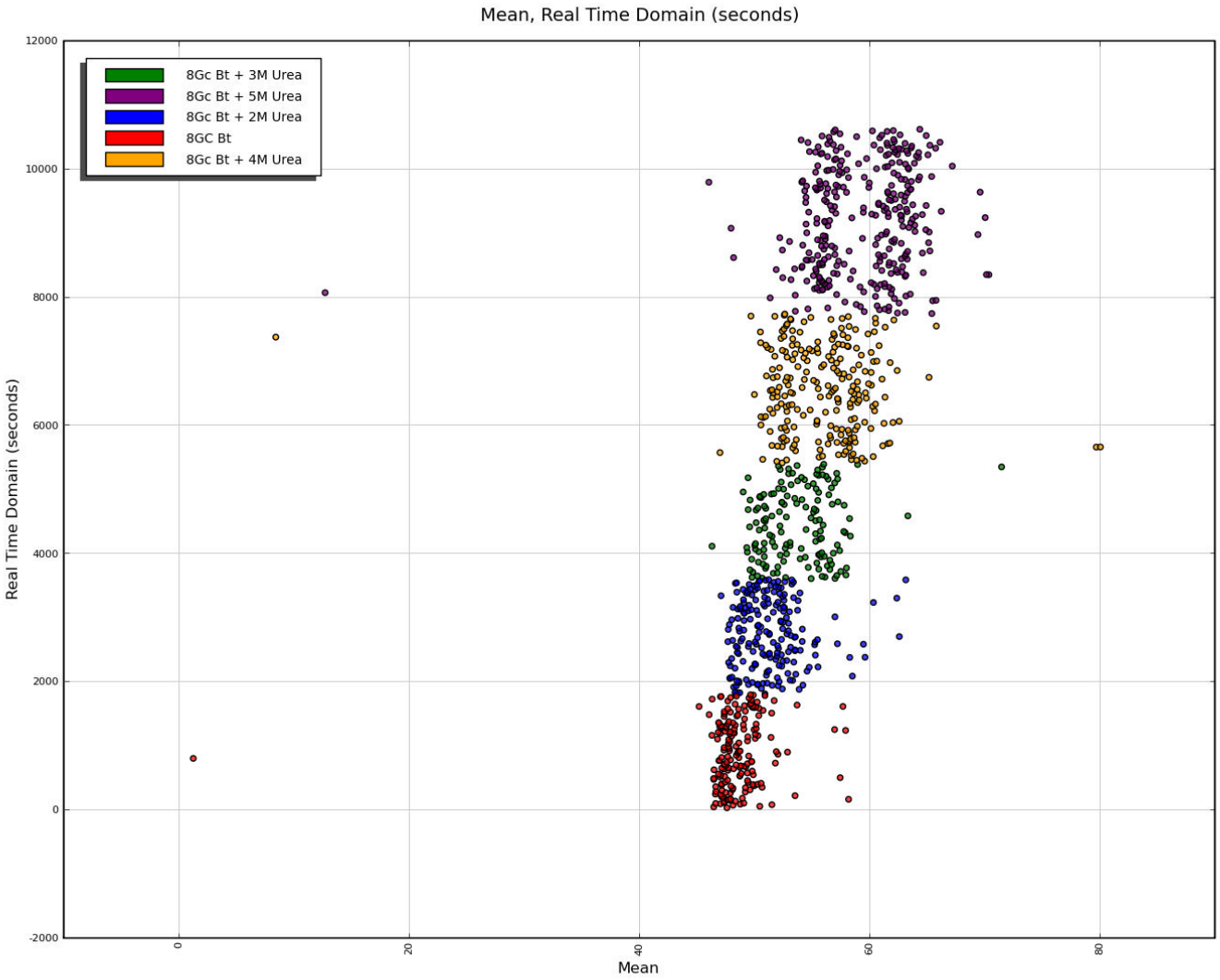

Mean, Real Time Domain (seconds)
8Gc Bt + 3M Urea
8Gc Bt + 5M Urea
8Gc Bt + 2M Urea
8GC Bt
8Gc Bt + 4M Urea
Real Time Domain (seconds)
Mean
12000
10000
8000
6000
4000
2000
0
-2000
0
20
40
60
80

Fig. 9.