Idaho Incubation Fund Program

Final Report Form

Proposal No.	**IF18-005**
Name:	**Dr. Daniel Fologea**
Name of Institution:	**Boise State University**
Project Title:	Engineered Advancements in Measuring Molecular Interactions in Support of Local Bio-industry

Information to be reported in your final report is as follows:

1. Provide a summary of overall project accomplishments to include goals/milestones met, any barriers encountered, and how the barriers were overcome: p1

   Specific outcomes: p1

2. Describe the current state of the technology and related product/service: p6

3. List the number of faculty and student participants as a result of funding: p7

4. What are the potential economic benefits: p7

5. Description future plans for project continuation or expansion: p7

6. Please provide a final expenditure report (attached) and include any comments here: p7, p9

7. List invention disclosures, patent, copyright and PVP applications filed, technology licenses/options signed, start-up businesses created, and industry involvement: p8

8. Any other pertinent information: p8

1. **Summary of overall project accomplishments that include goals/milestones met, any barriers encountered, and how the barriers were overcome. Specific outcomes.**

Goal 1. Coat glass beads with artificial membranes and test their interactions. This goal was achieved by producing artificial membrane systems around large glass beads (~50 µm diameter) to be used in the KinExA instrument by using the procedure developed in our lab specifically for this grant and described in the progress reports. After bead preparation, their ability to bind the FITC-Cholera Toxin B (FITC-CTB) was assessed by fluorescence microscopy (Fig. 1a, b). Next, we tested the beads for binding/unbinding in the flow cell of the KinExA instrument; our results (Fig. 2) demonstrate that the functionalized beads bind the fluorescent ligands, therefore enabling quantification of protein-membrane interactions by using artificial bilayer lipid membrane systems.

A B

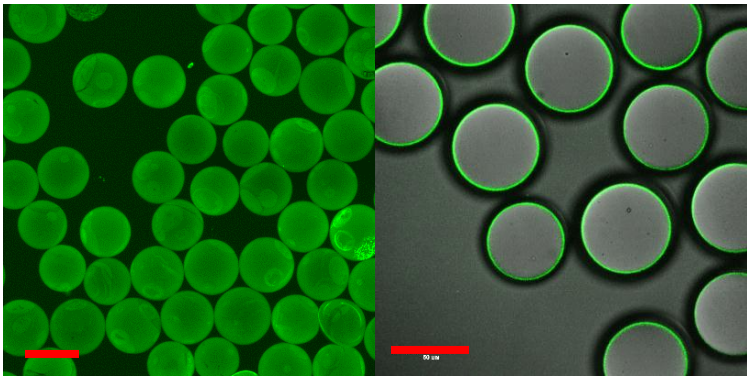


Fig. 1 Microscopic verification of bead-supported membranes. A) The lipid membranes supported by glass beads were imaged by fluorescent labeling of the membrane and wide-field fluorescence microscopy. b) GM1-membranes supported by glass beads are targets for FITC-CTB, as indicated by confocal imaging (scale bar = 50 µm).

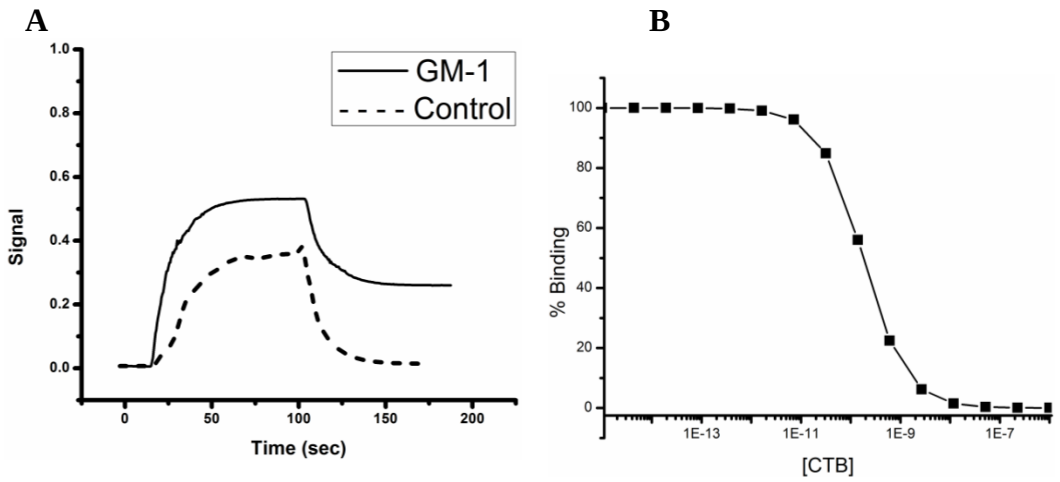


Fig 2. Binding assessment of CTB to functionalized glass beads - KinExA measurements. A) GM-1 (CTB-target) containing lipid membranes bind to Alexa Fluor 647 CTB as indicated by the end binding signal, indicative of capture. Absence of fluorescent end signal, indicative of no capture, was observed for membranes lacking GM-1 (Control). B**)** The end binding signals in equilibrium experiments (titrated CTB) were plotted and analyzed with the KinExA Pro software. The best fit provided a K_d value of 176 pM.

Goal 2: Coat glass beads with cell membranes, and Goal 3: Quantify specific antibody affinity for cell surface antigens with KinExA.

The membrane preparation procedures, specific to the project, were developed and reported during the project. Briefly, the cells were mixed with the glass beads, subjected to four freeze-thaw cycles, and sonicated in a water-bath sonicator for four minutes. To test the binding with the KinExA instrument, we proceeded by using the RBC-coated glass beads and FITC conjugated antibodies capable of specifically binding components of the RBCs. For the Jurkat membrane beads, competing non-labeled and labeled mouse anti-HLA antibodies were used as a model system. After reconstitution of membrane systems with glass beads as support, the RBC membrane functionalized beads showed strong interactions with anti-sheep fluorescent antibodies (Fig. 3).

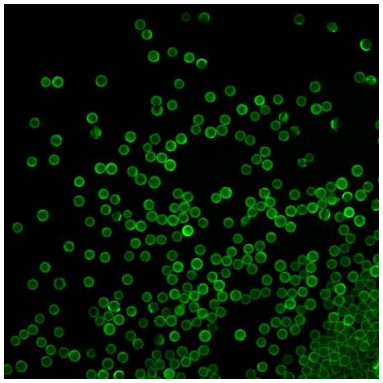


Fig 3. Microscopy image of natural membranes reconstituted on glass beads (50 μm diameter) with precursor sheep RBC shows interactions with cognate ligands (fluorescent anti-sheep antibodies, FITC-labeled).

The RBC-functionalized bead samples were used for kinetics measurements in the KinExA instrument. The evolution of the fluorescent signal following introduction of fluorescent ligands into the flow cell, followed by removal of free labels, enabled direct determination of the affinity. The signal recorded at the end of each tail (Figure 4a), proportional to the amount of bound antibodies, was converted into percent of bound ligands (occupied sites) relative to the total number of binding sites and plotted as a function of concentration (Fig. 4b) as average values from three experiments. Further analysis performed by assuming an isothermal binding process yielded a K_d value of 48.56 nM ± 3.18%.

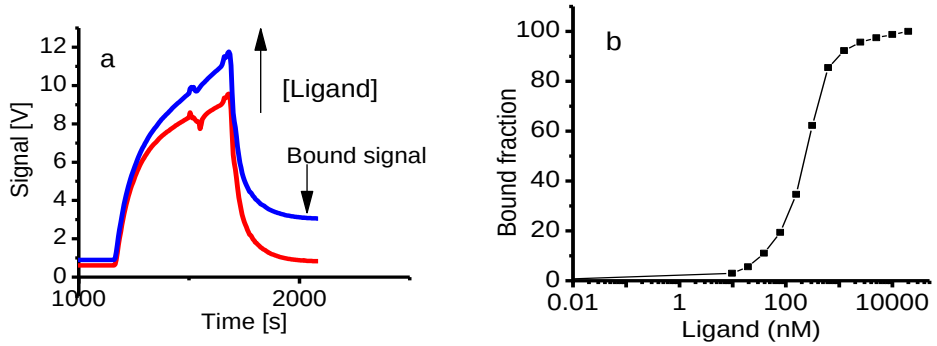


Fig. 4. a) KinExA instrument readout of specific antibody binding to sheep RBC membrane beads for two differentligand concentrations. b) Consolidated data used to determine K_d.

We also used Jurkat membrane coated beads, non-labeled mouse anti HLA antibodies (constant binding partner, CBP), and varied amounts of fluorescent mouse anti HLA antibodies (titrant). The membrane-covered glass beads were simultaneously exposed to a mixture containing both fluorescent and non-fluorescent labels, which competed for the available binding sites. The binding curve constructed from the average percent free CBP as a function of non-labeled titrant concentration was analyzed with the software package included with the instrument (Figure 5). The functional affinity constant K_d was calculated from the best fit of the plot (52.90 pM ± 2.43%).

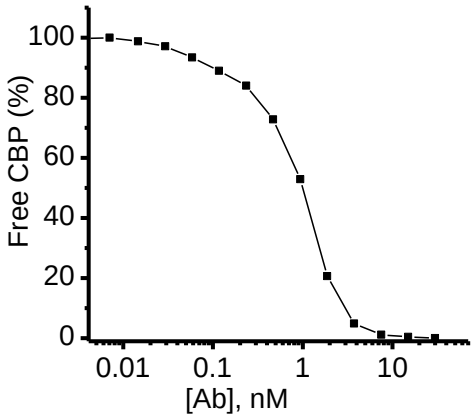


Fig. 5 Affinity quantification with the KinExA instrument for anti HLA antibodies targeting Jurkat cell membranes reconstituted on glass beads.

Goal 4. Investigate the stability of glass-bead supported membranes.

Glass bead batches were prepared by following the procedure described for natural cell membranes. Asolectin and cholesterol (building blocks of artificial membrane systems) were dissolved in chloroform, and 20 mg glass beads were immersed in the lipid mixture (200 µL) in a glass vial. The organic solvent was evaporated by vacuum exposure for 24 hours and the lipid cakes hydrated with a 135 mM KCl solution buffered with 20 mM Hepes at pH 7.2. The hydration was facilitated by alternating freeze-thaw cycles. After five cycles, the beads were exposed to two sonication cycles (two minutes each) in a bath sonicator for supported membrane formation around the glass beads. The beads were then washed by five cycles of centrifugation, with supernatant removal and buffer exchange at each cycle. The prepared beads were tested for artificial membrane presence by fluorescence microscopy imaging analysis after addition of fluorescent lipophilic compounds. Next, hydrated beads (no lipophilic dyes) were stored in a refrigerator for 6 months and re-tested with lipophilic fluorescence dyes (HF) via confocal fluorescence microscopy for confirmation of bilayer membrane integrity (Fig. 6).

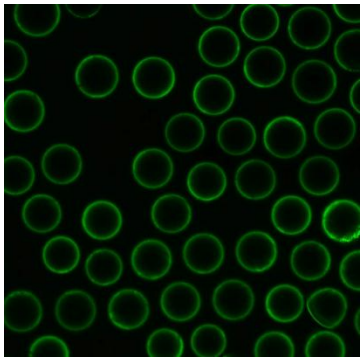
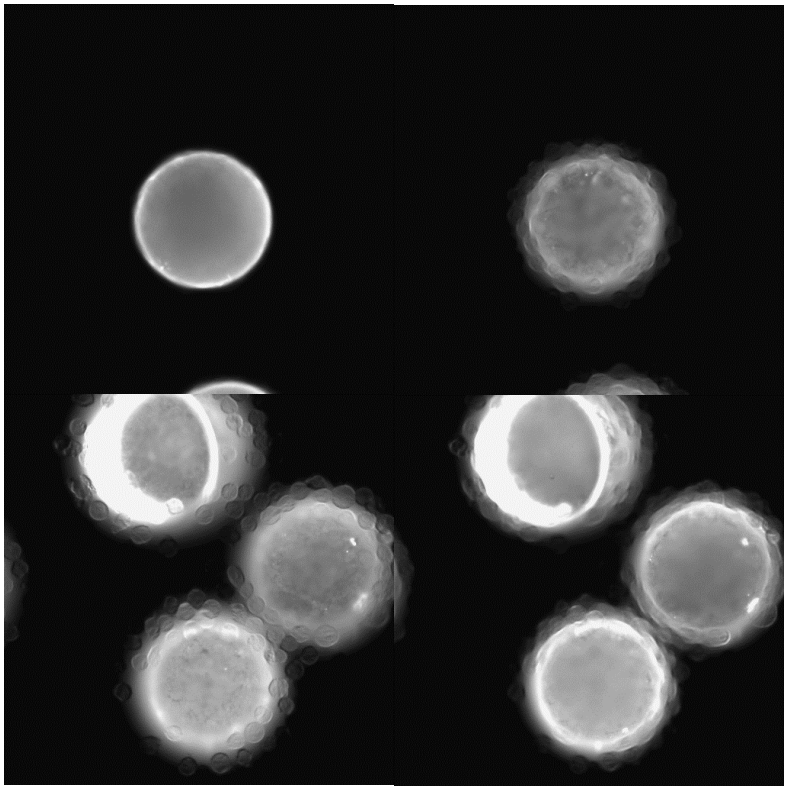


Fig. 6 Extended stability of glass bead supported artificial membranes. Fluorescent image of supported artificial membrane system after addition of membrane probe HF, with average bead diameter of 135 µm. The supported membranes were reconstituted six months before staining with HF and analysis with confocal fluorescence microscopy.

Supplementary achievements and developments.

We succeeded to directly attach fixed Jurkat cells to the surface of the PMMA beads (identical to what is used for typical kinetics experiments) by using an FSL-biotin linker, along with anchoring Jurkat cells to the same beads via CTB. This novel approach (Fig. 7) provides a better way for determining the kinetics since the light weight of the PMMA beads precludes addition of glycerol to the running buffer, which may slow down the diffusion.



Set1

Fig. 7. Evidence of Jurkat cell immobilization on PMMA beads. The PMMA beads have been functionalized with BSA-biotin, and the Jurkat cells with FSL-biotin. The cross-linking was achieved with streptavidin bridges. Each micrograph set represents distinct focal planes of the same field, and facilitate observing the attached cells in limited field depth conditions.

Set 2

Barriers encountered, and how the barriers were overcome. Direct cell attachment to the PMMA beads is considered one of the most promising approaches developed for quantification of interactions between cell membranes and ligands. However, we encountered several problems with repeatability of kinetics experiments when storing the beads-attached cells for extended time. We concluded that the anchoring via FSL-biotin or CTB does not completely prevent cell detachment from the beads, therefore reducing the concentration of the binding partner. This problem was alleviated by a complete wash of the beads before experiments and buffer exchange. Removal of the non-attached binders eliminates cross-interactions in solution but reduces the sensitivity of the measurements for long-term stored beads. Also, we observed that the measurements with the KinExA instrument on bead-attached cells are more reliable when low flow rates are used. Although this is not a major roadblock, in some situations experiments may have to be performed in shorter times. We concluded that the large size of the attached cells yields significant drag forces, which may detach the cells from the supporting beads. To reduce the size of the attached entity, we propose to use instead small unilamellar liposomes produced from the target cell. This approach was verified by using red blood cell membranes as biological material extruded through 1 μm Nuclepore filters mounted in an Avanti Polar Lipids manual extruder.

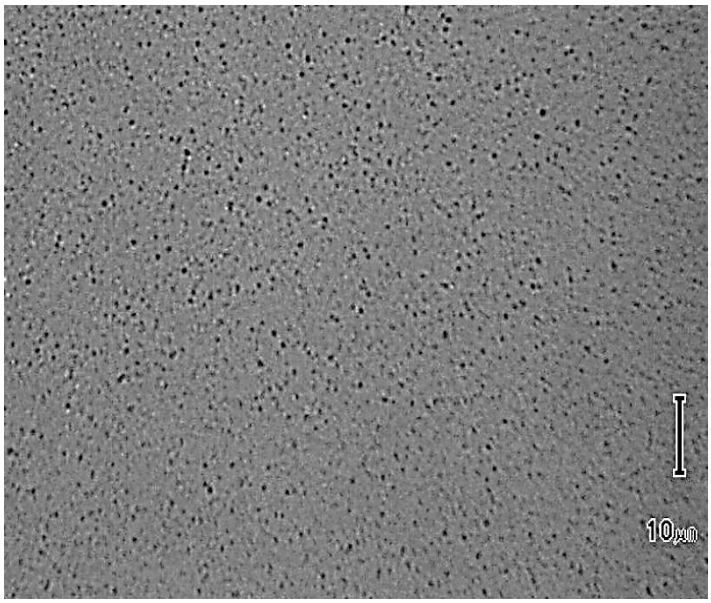


Fig. 8 Liposomes produced by extrusion of intact RBCs through 1 μm Nuclepore filters mounted in an Avanti Polar Lipids manual extruder.

The liposomes attached to the beads exhibited greater stability at higher flows in the KinExa microfluidic chamber; although this is a recent development, no change in their affinity to specific ligands (anti-sheep Ab) have been observed after an 8-week storage period.

2. Describe the current state of the technology and related product/service.

The one-year research project, developed in partnership with Sapidyne (commercial partner, producer of the KinExA instrument), resulted in several technologies to be used with the KinExA instrument for measuring membrane-ligand interactions, providing an unsurpassed advantage over competitors..

2.1 We developed **standard procedures for reconstitution of artificial membrane systems** by using glass beads as membrane support. These beads are further used to quantify the interactions between ligands and membranes with the KinExA instrument. We also succeeded in reconstituting specific transmembrane receptors (proteins) into the glass bead-supported lipid membranes, thereby substantially expanding the measuring capabilities of the instrument.
2.2 We developed **standard procedures for reconstitution of cell membranes (RBC, and Jurkat)** on glass beads, therefore displaying all the binding partners available on the live cell membranes. These functionalized glass beads may be further use to quantify any interaction with molecules or groups naturally presented on the live cell membranes.
2.3 We developed **standard procedures for attachment of intact cells on the PMMA beads,** thus increasing the surface area available for interactions and minimizing the effects of non-specific binding to the glass surface.
2.4 An unexpected development was provided by the necessity to produce small, uniform liposomes from cell membranes and artificial lipids for further attachment to PMMA beads and use with the KinExA instrument. The most common liposome extrusion system (produced by Avanti Polar Lipids), a manual one, includes lengthy and difficult preparation steps and extrusion protocols. After presenting Sapidyne with a prototype of an automatic extrusion system

developed at Boise State University, the company declared a great interest in licensing this technology and producing the extrusion system as stand-alone or an accessory to the KinExA instrument. More details are presented in section 5, future plans for project continuation and expansion.

3. List the number of faculty and student participants as a result of funding. This project directly funded one faculty (Fologea) and six undergraduate students (Andy Bogard, Colleen Poulton, Lizzie Leung, Jesse Schimpf, Daniel Prather, and Jessika Dagostino). Two more faculty (Dr. Denise Wingett, Co-PI, and Dr. Rebecca Hermannn) actively participated at research activities made possible by this project. In addition, two graduate students (Marcelo Ayllon, and Mark Smith) are working towards their PhD thesis using the technologies made possible through this grant. Six more undergraduate students (volunteers, or enrolled in research classes) and three volunteer high school students were engaged in research activities related to this project. **A total of 3 faculty members, 2 graduate students, 12 undergraduate students, and 3 high school students participated at research activities made possible through this project.**

4. What are the potential economic benefits: This project demonstrated that the technical capabilities of the KinExA instrument can be significantly expanded to quantification of membrane-ligand interactions in both artificial and natural membrane systems. The proposed technologies do not imply any modifications of the instrument but uses membranes reconstituted by using either glass or PMMA beads as support. The functionalized beads are further introduced into the microfluidic system of the instrument, and all the measurements are performed, recorded, and analyzed using the included program. Such advancement and addition of unique features to the existing KinExA instrument is anticipated to lead to a great advantage over competition and improved marketability by providing novel capabilities of investigating the functionality of biological systems in health and in disease, diagnosis, and drug development (see section 8 - other pertinent information - for more details).

5. Description future plans for project continuation or expansion. The research team is actively seeking a continuation of the research initiated through this project. In partnership with Sapidyne, Boise State University intends to develop an automatic liposome extrusion system, which is a much-needed device for liposome production using either lipid mixtures or cell membranes as starter materials. This technology will be licensed to Sapidyne, which will produce and commercialize it as stand-alone equipment or accessory to their instrument.

6. Please provide a final expenditure report (attached) and include any comments here. The final expenditure report is included at the end of this final report. Each budgetary category was fully spent for research activities related to this project.

7. List invention disclosures, patent, copyright and PVP applications filed, technology licenses/options signed, start-up businesses created, and industry involvement. After discussions with Sapidyne and the Technology Officer at Boise State University, we concluded that public dissemination of the novel technologies for measuring membrane-ligand interactions with the KinExA instrument is the best approach to promote the instrument, its novel capabilities, and to boost the sales of equipment and accessories. Several steps towards efficient dissemination have been taken, as detailed in the next section. This option is also justified by the fact that the newly developed technologies may not be used for measuring interactions by any other competitor instrument, and Sapidyne holds all the patents related to KinExA. However, after discussing with Sapidyne and the Technology Transfer Officer at Boise State, we decided to prepare a disclosure for the automatic extruder system, which will be prepared and patented by Boise State University. Once the patent application is filed, Sapidyne will license the technology from Boise State University, produce, and commercialize the device.

8. Any other pertinent information:

8.1 Applications of the novel technologies for measuring ligand-membrane interactions, as well as other interactions of great scientific interest have been presented by the PI and his students at the Biophysical Society Conference in 2018, a large scientific venue comprising several thousand attendants. These presentations raised a large interest from the audience, and these advancements have been highly recommended through scientific media.

8.2 The PI was invited to present these advancements in few seminars at the University of Bucharest, National Institute of Physics and Nuclear Engineering, and the National Conference of Biophysics (all in Romania). These events comprise a large attendance from many European countries. Further use of the technologies in those countries is feasible since Sapidyne has a European headquarters in Hannover, Germany.

8.3 Sapidyne discussed with existing and potential customers these novel scientific opportunities made possible through this project. Those customers are very interested in using these technologies for drug development and discovery, and immunology studies. However, they are interested in standard procedures for preparation of beads that comprise other cell lines than we used for our experiments in this project. Since Boise State University has the know-how for these technologies, these customers are interested in collaboration on establishing standard operational procedures for the cell lines of interest.

8.4 Two manuscripts related to these technologies and scientific advancement obtained with the KinExA instrument are currently under preparation; they will include authors from Boise State (faculty, undergraduate and graduate students) and scientists from Sapidyne who essentially contributed to this project. This dissemination pathway is considered one of the most effective ways for promoting the technologies developed at Boise State, the KinexA instrument produced by Sapidyne, and to acknowledge the financial support for this project from ISBOE.

FINAL EXPENDITURE REPORT

A. FACULTY AND STAFF		
Name/Title	$ Amount Requested	Actual $ Spent
Daniel Fologea / Associate Professor	7,867.00	7,867.00

B. VISITING PROFESSORS		
Name/Title	$ Amount Requested	Actual $ Spent

C. POST DOCTORAL ASSOCIATES/OTHER PROFESSIONALS		
Name/Title	$ Amount Requested	Actual $ Spent

D. GRADUATE/UNDERGRADUATE STUDENTS		
Name/Title	$ Amount Requested	Actual $ Spent
Andy Bogard, Colleen Poulton, Lizzie Leung, Jessika Dagostino, Daniel Prather, Jesse Schimpf -- students	16,840.00	17,191.00

E. FRINGE BENEFITS		
Rate of Fringe (%)	$ Amount Requested	Actual $ Spent
Fologea rate 38%	2,989.00	2,254.49
Student rate 4%	674.00	818.65
PERSONNEL SUBTOTAL:	**28,370.00**	**28,131.14**

F. EQUIPMENT: (List each item with a cost in excess of $1000)		
Item/Description	$ Amount Requested	Actual $ Spent
1.Biosafety cabinet	5,400.00	6,960.66
2.High resolution microscope camera	9,400.00	8,453.18
EQUIPMENT SUBTOTAL:	**14,800.00**	15,413.84

G. TRAVEL		
Description	$ Amount Requested	Actual $ Spent
1.		
2.		
3.		
TRAVEL SUBTOTAL:		

H. PARTICIPANT SUPPORT COSTS:

Description	$ Amount Requested	Actual $ Spent
1.		
2.		
3		
PARTICIPANT SUPPORT COSTS SUBTOTAL:		

I. OTHER DIRECT COSTS:

Description		$ Amount Requested	Actual $ Spent
1.Materials and supplies		20,300.00	24,876.29
2 Computers (2)		4,200.00	1,937.49
3.CO2 incubator		4,500.00	3,786.98
4. Recharge Center charges		2,800.00	823.97
OTHER DIRECT COSTS SUBTOTAL:		31,800.00	31,427.73
TOTAL COSTS (Add Subtotals):		74,970.00	74,969.71
TOTAL AMOUNT REQUESTED:			74,970.00
TOTAL AMOUNT SPENT:			74,969.71