GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION
SPONSORED PROJECT INITIATION

Date: May 22, 1979

Project Title: Genetics of Thermal Acclimation, Membranes and Aging

Project No: G-32-B02 (Green card)

Project Director: Dr. David B. Dusenbery

Sponsor: DHEW/PHS/NIH - National Institute on Aging

Agreement Period: From 5/1/79 Until 4/30/80 (02 Year)

Type Agreement: Grant No. 5 R01 AG00942-02

Amount: $70,941 New PHS Funds (G-32-B02)
3,735 GIT Contribution (G-32-326)
$74,676 Total

Reports Required: Annual Progress Report with Continuation Applications
Terminal Progress Report upon Grant expiration

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NOTE: Follow-On Project to G-32-B01 (01 Year).

Defense Priority Rating: N/A

Assigned to: Biology (School/Laboratory)

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CA–3 (3/76)

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION

Date: April 17, 1980

Project Title: Genetics of Thermal Acclimation, Membranes and Aging

Project No: G-32-B02

Project Director: Dr. David B. Dusenbery

Sponsor: DHEW/PHS/NIH - National Institute on Aging

Effective Termination Date: April 30, 1980 (End of 02 Year)

Clearance of Accounting Charges: ---

Grant/Contract Closeout Actions Remaining:

_ Final Invoice and Closing Documents
_ Final Fiscal Report
_ Final Report of Inventions
_ Govt. Property Inventory & Related Certificate
_ Classified Material Certificate
x Other Annual Report of Expenditures due by 7/31/80

NOTE: FOLLOW-ON PROJECT (03 YEAR) IS G-32-B03

Assigned to: Biology (School/~~Laboratory~~)

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CA—4 (1/79)

C. Progress Report

1. Introduction

This report covers the two years from May, 1978 to May, 1980. The "Specific Aims" of the previous proposal included:

1) Measurement of thermal acclimation and of the change (if any) of the ability to acclimate during aging. Progress on these aims are discussed in Section 3.

2) Isolation and characterization of mutants altered in thermal acclimation, which is discussed in Section 4.

3) Identification of membrane properties which change with acclimation temperature. Studies of polar lipid viscosity, which appears to have this property, are discussed in Section 5.

4) The effects of thermal adaptation mutants on lipofuscin formation. The development of methods to quantitate and characterize lipofuscin is discussed in Section 6.

An unappreciated problem in carrying out several of these studies was the difficulty of obtaining cultures of age synchronized C. elegans. We wanted to avoid the use of drugs and explored a variety of screening techniques. These explorations are described in the following section.

2. Age Synchrony

A variety of techniques for synchronous growth of C. elegans are described in the literature. While inhibitors of DNA synthesis have proven useful in maintaining synchrony in large-scale preparations (Mitchell et al, 79), they have inherent disadvantages. Synchrony can be maintained, without exogenous chemical agents, by means of physical separation. We developed a selective screening technique to maintain synchrony of worms cultured monoxenically on NGM agar and E. coli.

Synchronous cultures are started from first stage larvae obtained by filtering unsynchronous stock cultures through Nitex 10μm screen. First stage larvae, synchronous to within ten hours, are used to innoculate petri plates containing 5 ml NGM agar innoculated with E. coli. If required tighter synchrony can be achieved by collecting worms which hatch from eggs over a shorter period. Reducing the volume of agar greatly reduces burrowing and thus increases worm yield. After approximately 64 hours growth at 25°C worms are filtered on 400 mesh stainless steel screen. This filter retains worms longer than 750 μm, i.e. virtually all adults plus some L4 under our growth conditions. The filtered worms are transferred to freshly prepared petri plate cultures and grown for up to 21 days at 15°C. Progeny are eliminated from synchronous cultures by filtering at 2 day intervals on the 400 mesh filter. Eggs can occasionally hatch before being laid and the larvae can remain inside the adult for several days. Endotokic worms could potentially cause asynchrony, but have not been a problem in our cultures, presumably because its occurance in monoxenic petri plate cultures is low. This procedure provides synchronous populations at yields sufficiently high for our studies of locomotor activity and microviscosity.

3. Thermal Acclimation

In a study previously published we were able to evaluate the temperature-activity relationship in C. elegans using long exposure (1 second duration) photo-

graphy (Dusenbery et al, 78). Thermal acclimation of locomotor activity was demonstrated in the sense that the temperature-activity relationship was shifted to lower temperatures by growth in the cold. In view of known age dependent changes in response to thermal stress, it seemed worthwhile to evaluate the influence of age upon the temperature-activity relationship in C. elegans.

Synchronous populations of C. elegans were started as described earlier and maintained at 15°C for 6, 14 or 20 days. The temperature-activity relationship for worms at each of these ages were determined 6 to 9 times and are shown graphically in Figure 1. There are marked reproducable age dependent changes in the temperature-activity relationship. The temperature-activity relationship of 6 day worms is similar to that previously described for asynchronous cultures grown at 15°C. With increasing age, activity at all temperatures is reduced. This is consistent with previous observations of age dependent changes in activity (Croll et al, 77). These experiments demonstrate that there is little if any change in the lower thermal limit of activity.

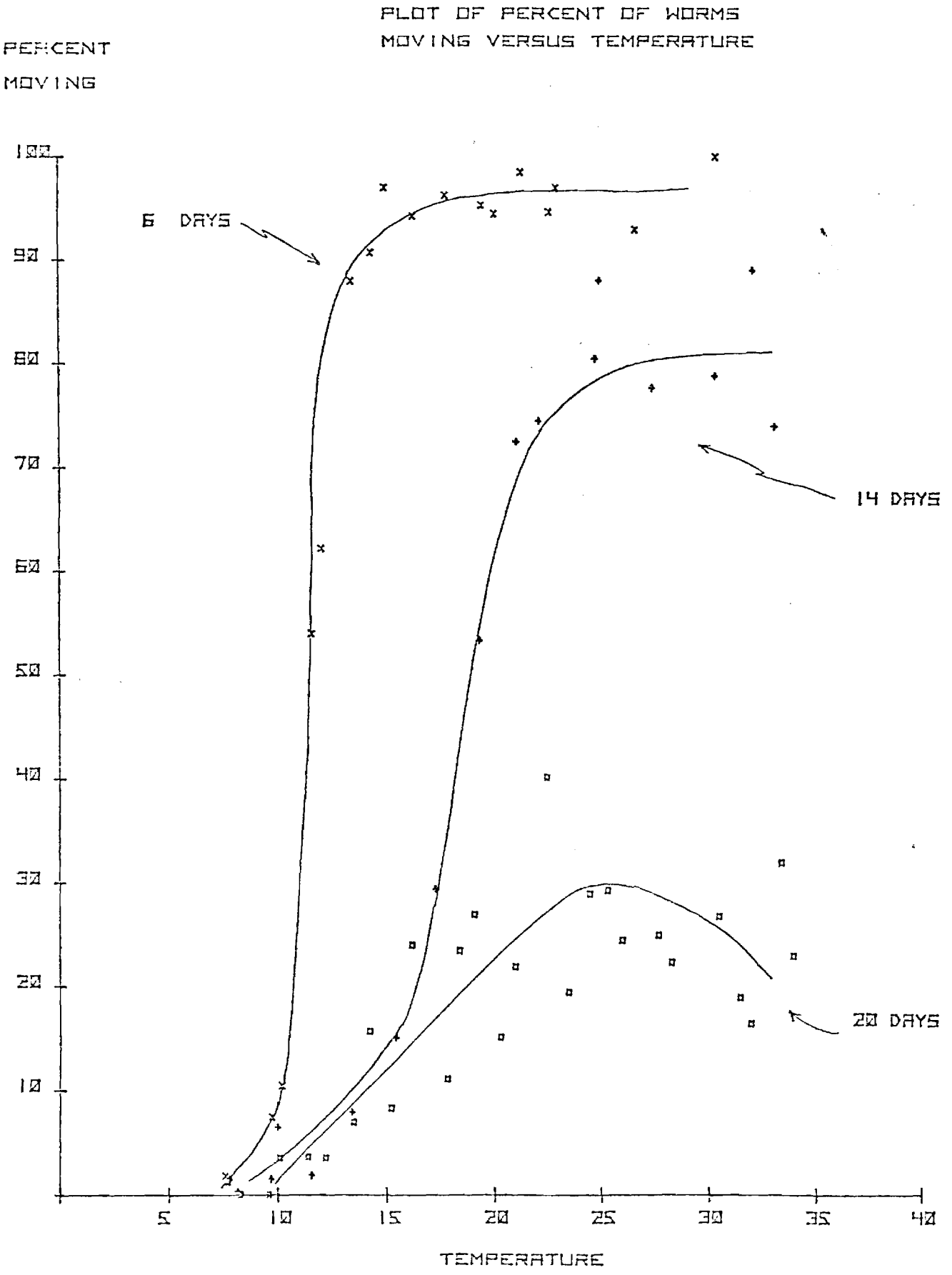
4. Cold Adapted Mutants

In order to explore mechanisms of thermal adaptation and to generate C. elegans strains with altered membrane lipid, an attempt was made to isolate mutant strains that were more active in the cold than the wild type. (Such strains are called 'cold adapted' here.) The wild type, N2, when grown at 25°C and tested for chemotaxis to NaCl at a nominal temperature of 10°C by countercurrent separation, responds very poorly. At most 1% of the worms will swim into the light layer containing NaCl. The majority sink into the dense layer. In contrast, about 90% of N2 grown at 15°C appear in the light layer (Dusenbery et al, 78). For the isolation of mutant strains, the N2 strain was mutagenized in the standard way. The mutagenized population was grown for several generations at 25°C in order for recessive mutations to become homozygous. These worms were then subjected to countercurrent separation at 10°C with NaCl in the light layer. All worms that exited the apparatus in the light layer were recultured for another generation or so and the isolation procedure repeated. After several rounds of alternate isolation and growth, several populations responded with a much higher fraction swimming into the light layer. Individual hermaphrodites from such populations were used to establish clones which were subsequently tested in the same way. Clones with the most abnormal behavior were chosen and used to start a strain. Not more than one strain originated from each mutagenized population, so that each strain arose from independent mutational events. Altogether ten such strains have been isolated.

In order to explore the thermal alterations exhibited by these strains, several measurements have been performed. Countercurrent separation tests have been made with several combinations of growth and test temperatures. Each strain and temperature combination has been tested 4 times. Under the conditions used to isolate the strains (growth 25°, test 10°) the means for each strain fell within the range 30 to 50% in the light layer whereas the wild type tested in parallel experiments was always $<1\%$.

In order to determine if the hot limit of swimming was shifted in addition to the cold limit, similar tests were performed at 30°C. With this temperature combination (growth 25°, test 30°C) the wild type makes substantial responses (50 to 70%). The response of several cold adapted strains was somewhat lower than that of N2 run in parallel but only one strain was significantly lower. This suggests but does not prove, that at least some of these strains have their whole range of thermal adaptation shifted to lower temperatures.

Figure 1


PLOT OF PERCENT OF WORMS
MOVING VERSUS TEMPERATURE
PERCENT
MOVING
100
90
80
70
60
50
40
30
20
10
6 DAYS
14 DAYS
20 DAYS
5
10
15
20
25
30
35
40
TEMPERATURE

Another important question was whether high growth temperature was required for the alteration of behavior to be expressed. Experiments parallel to those above were performed on wild type and mutant strains grown at 15°C. The results were generally similar to those with the higher growth temperature. Thus these strains do not appear to be temperature sensitive mutants in the usual sense. That is, growth at high temperature is not necessary to express the mutation.

Some of these strains have also been characterized by a different technique. It determines the fractions of worms in a population that are moving at an instant as the temperature is increased from about 5 to 35°C (Dusenbery et al, 78). For N2 grown at 25°C, at most a few percent move below 10°C. At higher temperatures the fraction moving increases rapidly, passing 50% at about 16°C, to a maximum of 90 to 100% between 20 and 25°C. Above 25° the fraction moving declined, passing 50% at about 30°C.

Several of the cold adapted strains have been characterized by this procedure. Data for the three strains which are best characterized are presented in the table titled "Thermal Limits." Five experiments were performed on each mutant strain on 5 different days and N2 was also tested on each of those days. If the mutant strain had a lower thermal limit that the wild type tested on the same day five out of five times, it was concluded that the mutant had a lower thermal limit than the wild type. As indicated in the table, two of the strains (A3 and A8) were significantly lower than N2 in both hot and cold limits. The cold limits were shifted about 2°C and the hot limits about 4°C. Strain A5 was lower in the hot limit (by about 2°C) but not lower in the cold limit. Thus it appears that at least two and probably more of these strains behave as if their thermal range of activity has been shifter to lower temperatures.

These strains are interesting from several points of view. The fact that in the cold they are more active than the wild type rather than less active suggests that the alterations causing the phenotype are more likely to involve processes important to thermal adaptation. Whatever the mechanism involved, its elucidation would probably be an important contribution to understanding thermal adaptation. If our anticipated finding that the viscosity of membrane lipids is lowered in these strains is supported, they will also provide material to test the effects of membrane properties on various aging parameters.

5. Membrane Properties

A question relevant to our goal of understanding the interrelationships among thermal acclimation, aging and membrane properties is whether thermal acclimation involves changes in membrane viscosity. To answer this question we have begun to measure the viscosity of lipid vesicles prepared from polar lipids extracted from worms grown at 15° or 25°C. Microviscosity is measured by fluorescence polarization of diphenylhexatriene (DPH) (Shinitzky and Barenholz, 78). DPH is added to vesicle preparation to achieve a lipid to probe ratio of 500:1. Following a period of probe equilibration microviscosity is measured over the temperature range of 5 to 40°C. The viscosity of polar lipids extracted from worms grown at 15° can be compared to that or worms grown at 25°C for any temperature within the range of test temperatures. If thermal acclimation of membrane viscosity occurs we might expect viscosity to be higher at a given test temperature for worms grown at 25°C than for cold acclimated, 15°C grown, worms. While these experiments are still in progress, results to date indicate that growth temperature does influence polar lipid viscosity. The table titled "Polar Lipid Viscosity" summarized the results of all such experiments

Table: Thermal Limits

	Strain			
Limit	N2	A3	A5	A8
Cold	16.3 ± 0.4 (-/40)	14.4 ± 0.6 (5/5)	16.3 ± 0.5 (3/5)	13.7 ± 0.6 (5/5)
Hot	30.1 ± 0.7 (-/39)	26.4 ± 0.5 (5/5)	28.3 ± 0.7 (5/5)	26.4 ± 1.0 (5/5)

Mean ± S.D. (# experiments in which the thermal limit of a strain was less than that of N2 tested on the same day/total # experiments with a particular strain.) The thermal limit reported is the temperature (^{o}C) at which 50% of the worms are active.

Table: Polar Lipid Microviscosity

			Growth Temp. 15°C		Growth Temp. 25°C
			1.35		1.97
			1.61		1.88
Exptl. Temp.	15°		1.56		1.96
			1.76		
		$\bar{X}$	1.57	$\bar{X}$	1.94
		S.D.	0.17	S.D.	0.05
			0.92		1.24
	25°		1.10		1.12
			1.03		1.19
			1.09		
		$\bar{X}$	1.04	$\bar{X}$	1.18
		S.D.	0.08	S.D.	0.06

For each experimental temperature the higher growth temperature produced a viscosity that was significantly higher at the 5% level using the two-sample rank test.

completed to date. Microviscosity at either 15° or 25°C is lower for 15° grown worms than for 25°C grown worms. The apparent extent of acclimation is about 40%.

6. Lipofuscin

Methods are being developed to quantitate the accumulation of lipofuscin in C. elegans and to characterize its fluorescence spectrum more accurately than has previously been reported. In particular, a method is being developed to obtain what have been called total luminescence spectra (Giering and Horning, 77) or the emission-excitation matrix (Warner et al, 76). That is spectra in which the intensity of all relevant combinations of excitation and emission wavelengths are recorded. Recent developments in electronics have made such spectra feasible and it has simultaneously been recognized that such spectra should be particularly useful in characterizing mixtures. Since lipofuscin is a poorly defined substance and is likely to include a mixture of fluorescent products, it was decided that recording such spectra would be useful.

In order to accomplish this, stepping motors have been attached to the monochrometers of the SLM fluorometer and interfaced to a Hewlett-Packard 9815S calculator so that fluorescence spectra can be acquired automatically. Programs have been written to scan both excitation and emission wavelengths systematically and to present the data as a two dimensional plot with contours of equal intensity. An example is shown in the Figure titled "Fluorescence Spectra."

At present the exciting light is monitored by a rhodamine guantum counter (Yguerabide, 68) and the intensity expressed as a fraction of the exciting intensity. This procedure corrects the spectra for variations in exciting light intensity. In the future it is planned to use a MgO reflector (Benford et al, 48 a,b) to calibrate the spectral response of the detector so that spectra can be corrected for changes in sensitivity to emitted light as well. Thus accurate quantitation of the fluorescent intensity with all combinations of excitation and emission wavelengths will be possible.

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1 →	GRANT NUMBER
SECTION IV—SUMMARY PROGRESS REPORT	AG00942-03

PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT	
Dusenbery, David B.	FROM	THROUGH
NAME OF ORGANIZATION Georgia Institute of Technology	4/1/79	3/31/80

TITLE (Repeat title shown in Item 1 on first page)
Genetics of Thermal Acclimation, Membranes, and Aging. G-32-B02

1. List publications: (a) published and not previously reported; (b) in press. Provide five reprints if not previously submitted.
2. List all additions and deletions in professional personnel and any changes in effort.
3. Progress Report. (See Instructions)

Interim Progress

1. Objective

The basic objective of this project is to explore the interrelationships between thermal acclimation, membrane properties, and aging in the nematode Caenorbabditis elegans. The organism was selected in order to make use of special properties it possesses for the isolation of mutations that would be useful to understanding these interrelationships. Studies of this type have not previously been carried out on organisms such as nematodes with differentiated organ systems.

The goals for the present year were to complete development of methods for isolating polar lipids and lipofusein, and to use these methods to characterize the strains altered in thermal acclimation. Measurements of the ability of aged animals to acclimate to temperature changes were also planned.

2. Results

Procedures were developed for obtaining pure populations of old nematodes using selective screening. Synchronous cultures were started with first and second stage lavae obtained by filtration on a Nitex filter #10. Once worms had grown to the egg bearing stage synchrony was maintained by intermittent filtering on a stainless steel 400 mesh filter. Adult worms were retained and transfered to fresh petriplate cultures. We have maintained synchrony without drugs for up to 21 days using these techniques.

These procedures were used to investigate changes in the temperature dependence of locomotoractivity with age. It was found that the temperature dependence of locomotor activity was altered. The activity at growth temperature and the maximum activity within the temperature range 5 to 35°C decreased with age. Furthermore, the young worms (6 days) were more active at temperatures below their growth temperature than were aged worms (14 and 19 days).

The fluorometer was further refined to provide more detailed information. A refrigerated water bath was designed that provides rapid temperature changes of the sample under the control of a desk-top calculator. A program was written that allows for automatic collection of large amounts of data on fluorescence polarization as a function of temperature. Methods were developed for the preparation of phospholipid dispersions from extracts of polar lipids. Initial experiments on lipids extracted from the wild-type strain indicate that it does indeed shift membrane viscosity to maintain a constant value at different growth temperatures. These procedures are applicable to determining the capacity of old worms (taken from synchronous cultures) to maintain a constant membrane fluidity.

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3. Significance

Membranes are important to a great many cell functions and thus any increase in understanding them will have wide significance. For example membrane properties are important in the process of viral infection of a cell and membrane alteration may be important in tumor growth. Thus an increased understanding of membrane properties would likely aid in understanding the developing control measures for these very significant health problems even though the project does not involve them directly. Changes in membrane properties may also be an important component of aging, although this point seems not to have been well-studied. Much better demonstrated is the fact that the ability to maintain constant body temperature during exposure to changing environmental temperature declines in old age. This means that many body parts, particularly the limbs, are subject to varying temperatures and the effects of this at the cellular level becomes an important question, that this project can contribute to.

4. Goals

In the coming year we plan to begin determinations of the polar lipid viscosity vs. temperature relationship for the wild-type strain grown at different temperatures, for the mutant strains with altered thermal acclimation, and for senescent nematodes. Lipofusein fluorescence will be analysed by 2-dimentional spectra. And the mutant strains altered in thermal acclimation will be assayed to determine if they differ from wild-type in the lipofusein formed.

The undersigned agrees to accept responsibility for the scientific and technical conduct of the project and for provision of required progress reports if a grant is awarded as the result of this application.

Date

Principal Investigator