II Materials and Methods:

2.1 Materials

2.1.1 Enzymes and Antibodies

Enzymes

- Restriction enzymes were purchased from New England Biolabs, Inc (www.neb.com)
- T4 DNA ligase was obtained from Roche (www.roche.com)
- Phosphatase alkaline (AP) was a product of Roche
- Biotin ligase (BirA) was obtained from Avidity (www.avidity.com)
- Thrombin protease was purchased from Amersham Biosciences (www.amershambiosciences.com).

Antibodies

- Antibodies for Rho were purchased from Promega (www.promega.com)
- Antibodies for Biotin were purchased from Promega
- ExtrAvidin alkaline Phosphatase conjugate were purchased from sigma. (www.sigmaaldrich.com)

Micellanea

- Protease inhibitor cocktail tablets (Roche)
- Plasmid and DNA gel purification kits (Qiagen, www.qiagen.com)
- Glutathione-sepharose 4B beads (Amersham Biosciences, www.amershambiosciences.com)
- Soft Link Release avidin Resin (Promega)
- All other chemical reagent were purchased from Sigma

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- Column PD-10 were from Amersham Pharmacia Biotech
- Centricon Centrifugal Filter Devices were purchased from Amicon (www.millipore.com)
- Biochemicals were obtained from Sigma
- ECL Western blotting detection reagents were purchased from Amersham Biosciences
- MonoQ HR 5/5 column from Pharmacia Biosciences

2.1.2 Vectors and Oligos

- PMT4 vector contains synthetic rhodopsin gene, was obtained from T.P. Salkmar, Rockefeller University
- PMAL-C2X was purchased from New England Biolad
- PGEX-BirA was a gift from Dr. M. Kursar (MPIIB-Berlin)
- All oligonucleotides were synthesized by and purchased from TIB Molbiol. (www.tib-molbiol.de)

2.1.3 Cells and *E.coli* Strains

COS cells used for expression of Rho mutants, were obtained from ATCC. *E.coli* stain BL21 was used for amplification of protein, strain XL-1 blue and DH5α used for amplification of plasmid DNA, were purchased from Stratagene. (www.stratagene.com)

2.1.4 Solution and Buffers

LB medium: 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, pH 7.0. For preparation of LB agar plates, add 1.5% of agar to the LB medium.

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PBS: 0.8% NaCl, 0.02% KCl, 0.268% $Na_2HPO_4 \cdot 7H2O$, 0.024% NaH_2PO_4, pH 7.4

SDS-sample buffer: 50 mM Tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 100 mM DTT, pH6.8

TAE: 0.04 M Tris-Actic acid, 0.001 M EDTA

TBS: 20 mM Tris-HCl, 137 mM NaCl, pH 7.6

TBST: TBS + 0.1% Tween 20

TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Buffer B: 10 mM NaH_2PO4, pH 7.5, 1 mM ethylenediamine tetra-acetic acid, 1 mM dithiothreitol

buffer C: 50 mM imidazole, pH 6.8, 50 mM NaCl, 5% glycerol, 5 mM mercaptoethanol

Biotinylation Buffer: A: 0.5 M Bicine pH 8.3, **B:** 100 mM ATP, 100 mM MgOAc, 400 µM Biotin

BTP buffer: 20 mM BTP, pH 7.5

G_1 Buffer: 130 mM NaCl, 1 mM $MgCl_2$, 0.01% (w/v) DM, 20 mM BTP, pH 7.5

MBP Column Buffer: 200 mM NaCl, 20 mM Hepes, 5 mM $MgCl_2$, 1 mM EDTA

MBP Wash Buffer: 500 mM NaCl, 20 mM Hepes, 5 mM $MgCl_2$, 1 mM EDTA

MBP Elution Buffer: Column buffer 20 mM Maltose, 1 mM DTT

Soft Link Release avidin Resin Equilibration Buffer: 50 mM Tris-HClpH 8.0,
100 mM NaCl, 4 mM DTT, 0.03% DM

Soft Link Release avidin Resin Elution Buffer: Soft Link Release avidin Resin Equilibration Buffer supplemented with 5 mM Biotin

G Buffer: 0.13 M NaCl, 1 mM $MgCl_2$, 1 mM DTT, 20 mM Tris 20 mM BTP, pH 7.5

OG 50 Buffer: 0.15 M NaCl, 1 mM $MgCl_2$, 10 mM NaPh, 50 mM octylglucoside

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2.1.5 Equipment:

UV-visible Spectrophotometer: Cary 50 Bio, Varian (www.varianinc.com)

Fluorescence Spectrometer: Fluorolog II, SPEX (www.spexcsp.com)

Ultracentrifuge: Opt^{TM} TLX, Beckman (www.beckman.com)

IAsys Cuvette System : Thermo Lab (www.thermo.com)

Photometer: Eppendorf (www.eppendorf.com)

Lumi imager: Roche

Fast Protein Liquid Chromatography: Pharmacia.

2.2 Methods

2.2.1 Expression and Purification of BirA

2.2.1.1 Expression of The GST-BirA Fusion Protein

Plasmid pGEX-BirA was derived from PGEX-2T. The gene encoding BirA was subcloned into plasmid pGEX-2T at the restriction site of BamHI. For expression, plasmid pGEX-BirA was transformed into the *E. coli* stain BL21. Cells were grown at 37°C in Luria-Bertani medium supplemented with 100 µg/ml ampcillin in 600 ml volumes and protein expression was induced with 0.5 mM isopropyl β-D-thiogalactopryanoside (IPTG) at a OD of ~0.5. Cells were harvested 3 hours after induction by centrifugation and the cell pellet resuspended in cold phosphate-buffered saline (PBS).

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2.2.1.2 Purification of BirA and Removal of GST Moiety

Cell lysis. Cells were lysed by sonication on ice. The lysate was ultracentrifuged at 40,000g for 45 min at 4°C and the supernatant was filtered by Syringe Filter (0.45 µm).

Affinity chromatography. The supernatant was applied to a 1-ml Glutathione Sepharose 4B column and the column was washed 10 column volume with PBS buffer (pH 7.3), at a flow rate of 0.1 ml/min.

On-column cleavage of the BirA - GST fusion protein by thrombin. In order to remove the BirA protein from the GST affinity tag, the fusion protein was digested with the protease thrombin while the fusion protein was bound to Glutathione Sepharose. 1 ml of thrombin (80 units) was loaded on the column, the column was incubated 16 hours at 22 ^{0}C. Cleavage of the bound fusion protein eliminates the extra step of separating the released protein from GST because the GST moiety remains bound to the sepharose resin while the desired protein is eluted with PBS buffer.

Desalting on PD 10. The elution from Glutathione Sepharose was applied to a PD 10 column. The column was eluted with buffer B (10 mM NaH_2PO4, pH 7.5, 1 mM ethylenediamine tetra-acetic acid, 1 mM dithiothreitol), and the protein fraction with absorbance at 280 nm was collected.

Anion exchange chromatography on Mono Q. The fraction from desalting step were applied to a MonoQ HR 5/5 column using FPLC (fast protein liquid chromatography). The column was washed with 5 ml buffer B and eluted with 50 ml of a linear gradient of 0 M to 0.6 M NaCl. Fractions were collected at a flow rate of 0.5 ml/min.

Gel filtration chromatography on Superdex 200. Fractions with the largest protein peak from FPLC were concentrated by ultra filtration to 0.3 ml with centricon 30, then applied to a Superdex 200 HR 10/30 column. The column was equilibrated with buffer C (50 mM imidazole, pH 6.8, 50 mM NaCl, 5% glycerol, 5 mM mercaptoethanol) and eluted with the

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same buffer. The fractions were collected at a flow rate of 0.3 ml/min.

All procedures were carried out at 0-4°C. Finally, BirA obtained was stored at –80°C.

2.2.2 Expression and Purification of MBP-Bio

2.2.2.1 Construction of Plasmid pMAL-Bio

Plasmid for expression of MBP-Bio: the pMAL-C2X vector was double digested with BamHI and SalI at 37°C for 2 hours, allowing for the isolation by gel electrophoresis of the 6.6 kb fragment containing the full sequence of the malE gene. The degisted vector was purified using Qiagen Gel Extraction Kit. 2 pmol forward oligonucleotide and 2 pmol reverse oligonucleotide (see below) were mixed and heated to 90°C for 10 minutes and then cooled down slowly to room temperature for annealing. Thereafter, 20-50ng vector DNA and 100-200 ng oligonucleotide duplex were mixed for ligation for 5 minutes at room temperature using 1 unit of fast ligase in a total volume of 20 µl.

E.coli XL-1blue competent cells were transformed according to the standard protocols (Sambrook, et al., 1989) with 4 µl ligation mixture. Clones were selected from ampicillin-containing LB agar plates.

All constructs were sequenced to verify that the correct junctions were formed during plasmid construction.

Oligonucleotides Used:

Fw: 5'-- GATCCGGCCTGAATGATATCTTTGAGGCCCAGAAGAT
TGAGTGGCACGAGTAAG -3'

Rev: 5'- TCGACTTACTCGTGCCACTCAATCTTCTGGGCCTCAAAG
ATATCATTCAGGCCG-3'

* The restriction site is underlined

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2.2.2.2 Amplification and Purification of MBP-Bio

The plasmid pMAL-Bio was transformed into *E. coli* strain BL21 and cells were grown at 37°C in Luria-Bertani medium supplemented with 100 µg/ml ampcillin and 2 g/L glucose in 600 ml volumes. When cells were grown to 2×10^8 cells/ml (OD of ~0.5), IPTG was added to a final concentration of 0.5 mM and the temperature was reduced to 31°C. Cells were harvested 3 hours after induction by centrifugation at 4000 g for 30 minutes. Cells were resuspended in 50 ml Column Buffer. The sample was placed in an ice-water bath and sonicated with 10 pulses for 30 seconds, centrifuged again at 9000 g for 30 min. The supernatant was loaded onto an amylose column.

Then the column was washed with 400 ml Column Buffer with a flow rate of 1ml/ minute and the fusion protein was eluted. with Column Buffer plus 10 mM maltose. 10 fractions of 5 ml each were collected and protein concentration was determined by Bradford protein assays

2.2.3 Expression and Purification of Recombinant Rhodopsin

2.2.3.1 Plasmids Used for Expression of Recombinant Rhodopsin

Oligonucleotides for pMT-Bio1:

Fw: 5´-- AATTCCACCATGGGCCTGAATGATATCTTTGAGGCC
CAGAAGATTGAGTGGCACGAGATGAACGGTAC --3'.

Rev: 5'--CGTTCATCTCGTGCCACTCAATCTTCTGGGCCTCAAAG
ATATCATTCAGGCCCATGGTGG--3'.

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Oligonucleotides for pMT-Bio2:

Fw:

5'--AATTCCACCATGGGCCTGGCTGATATCTTTGAGGCCCA
GAAGATTGAGTGGCATGAGGGTAC --3'

Rev:

5'-- CCTCATGCCACTCAATCTTCTGGGCCTCAAAGATATCAG
CCAGGCCCATGGTGG --3'

Oligonucleotides for pMT-Bio3:

Fw: 5'-- TCGACCGGCCTGAATGATATCTTTGAGGCCCAGAAG

ATTGAGTGGCACGAG --3'

Rev: 5'-- TCGACTCGTGCCACTCAATCTTCTGGGCCTCAAAGA

TATCATTCAGGCCGG --3

* The restriction site is underlined

PMT4 was used as the parent plasmid for construction of the plasmids pMT-Bio1, pMT-Bio2 and pMT-Bio3, which contain the biotinylation consensus domain at the N-terminus of rhodopsin or close to the C-terminus (pMT-Bio3). The PMT4 vector (containing the synthetic rhodopsin gene) was digested with KpnI and EcoRI to remove a 63 bp fragment including the rhodopsin start codon. The digested vector (6.14 kb) was isolated by gel electrophoresis. For PMT-Bio3, the PMT4 vector was digested with Sal I and isolated by gel electrophoresis. The digested vector was dissolved in 50 µl TE (pH 7.5), and dephosphorylated with Alkaline Phosphatase (1µl). Deionized water (39µl) and reaction buffer (10µl), has added and the mixture incubate at 37°C for 30 minutes. The reaction was stopped by heating to 65°C for 15 minutes and DNA extracted with the Qiaquick Gel Extraction Kit (Qiagen).

The vectors were then ligated with anealed oligonucleotides using quick T4 DNA ligase for 5 minutes at room temperature (for vector maps see Fig. 17, §3.2.3).

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2.2.3.2 Expression and Preparation of Recombinant Rhodopsin:

Expression of opsin genes was performed basically as described by Meyer et al. (2000): COS-1 cells were grown in monolayer in plastic cell culture roller bottles (surface area 850 cm^2) under a humidified atmosphere with 5% CO_2 at 37 °C. Growth medium was Dulbecco's modified Eagle's medium containing 4.5 g/liter D-glucose and 0.11 g/liter sodium pyruvate, supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine, and 10% (v/v) heat-inactivated fetal bovine serum. At 80-95% confluence, transient transfection of the cells was initiated using a transfection mixture composed of 150 µg of plasmid DNA, 6 ml of 1 M Tris buffer (pH 7.4), 48 ml of serum-free growth medium, and 6 ml of DEAE-dextran stock solution (2.5 mg/ml in Dulbecco's modified Eagle's medium). After 5.5 h, the transfection mixture was replaced with 75 ml of 0.1 mM chloroquine in growth medium for 90 min. The cells were washed with Dulbecco's modified Eagle's medium and further incubated in growth medium until harvest 64-68 h later. By incubation with 1 mM Na_2EDTA in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.0) for 10 min, cells expressing the apoproteins were detached from the plastic surface. The cell suspension was supplemented with protease inhibitors and incubated with 30 µM (final concentration) 11-*cis*-retinal for 2-4 h at 4 °C to reconstitute the pigment. The rhodopsins were purified by immunoaffinity adsorption based on a procedure described by Oprian *et al.* (1987). Briefly, cells were solubilized by the addition of dodecyl maltoside (1% (w/v) final concentration), and nuclei were removed by centrifugation. Pigments from two roller bottles were incubated with 1D4-Sepharose and washed twice with 0.03% (w/v) DM in phosphate-buffered saline and once with BTP buffer (0.03% (w/v) DM, 10 mM BTP, pH 6.0; 50 ml/wash). Elution of the pigments from the 1D4-Sepharose was carried out in BTP buffer supplemented with 100 µM peptide corresponding to the carboxyl-terminal 18 amino acids of rhodopsin.

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Elutions were pooled and concentrated to about 10µM using an Amicon Centricon 30 concentrator.

2.2.4. Preparation of Transducin

Gt holoprotein was purified from rod outer segment preparations essentially as described previously (Heck, et al., 1992). Rod outer segments were purified from fresh dark-adapted bovine retinas obtained from a local slaughterhouse using the discontinuous sucrose gradient method described by Papermaster (Papermaster, 1982). Retinas were dissected, and rod outer segments were isolated under dim red illumination. All subsequent procedures were performed at 0-5 °C, and the rod outer segments were stored frozen at 80 °C until used.

Preparation of Washed Membranes: Rhodopsin was prepared by removing the soluble and membrane-associated proteins from the disc membrane by repetitive washes with a low ionic strength buffer (Kühn, 1982). All purification steps were performed under dim red illumination, and the membrane suspension was stored at 80 °C until used.

Preparation of Transducin: Transducin was isolated from frozen dark-adapted bovine retinas according to Heck and Hofmann (Heck, et al., 1993).

2.2.5. Biotinylation of Recombinant Proteins

The biotinylation reaction was performed as following: recombinant rhodopsin or MBP-Bio (concentration about 10 µM) were buffer exchanged into BirA reaction buffer (0.05 M Bicine pH 8.3, 10 mM ATP, 10 mM MgOAc, 0.03% DM) with 100 µM Biotin. Recombinant BirA enzyme (5 µM) and protease inhibitor (0.1 mM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin, and 1 µg/ml pepstatin) were added, and the reaction was

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incubated at room temperature overnight.

2.2.6. SDS-PAGE and Western Blotting Analysis

Proteins from the biotinylation reaction mixture were solubilized in SDS-sample buffer, heated at 95°C for 2 minutes and centrifuged for 2 minutes at 10,000g. The clear lysates were loaded and separated by discontinous SDS-PAGE under reducing conditions (Sambrook et al., 1989) using a separating gel containing 12% of bisacrylamide/acrylamide, respectively, at a ratio of 1:29. Gels were stained with Coomassie brilliant blue R 250. For Western blotting, proteins were electrophoretically transferred onto NC membranes (Protran BA85, 0.45µm). The NC membranes containing transferred proteins were blocked for 30 minutes with 5% defatted milk solubilized in TBST buffer, then washed with TBST and incubated with primary antibodies for two hours. Immunoreactive bands were detected with an appropriate secondary antibody conjugated to HRP. After each step, the membranes were washed three times with TBST. Membranes were developed with ECL Western blotting detection reagents and detected using Lumi-Imager (Roche).

2.2.7 Quantitation of Biotinylated Rhodopsin

Biotinylated rhodopsin bind to a streptavidin resin with strong affinity, the K_D in the order of 10^{-15}. Streptavidin sepharose was used to quantitate the amount of biotinylated rhodopsin. After the biotinylation reaction the mixture was applied to streptavidin agarose. Only biotinylated rhodopsin binds to it, non-biotinylated rhodopsin remains in the flow-through. By making use of the absorption of rhodopsin, which has a maximal absorption at 500 nm (ε_{500} = 42700 $M^{-1}cm^{-1}$), the amount of rhodopsin which did not bind can easily be

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measured. The amount of rhodopsin in the flow through and different wash steps was determined from the spectra and the difference to the sample applied was calculated.

Binding to streptavidin resin: 200µl rhodopsin (biotinylated and unbiotinylated rhodopsin) was mixed with 50µl streptavidin resin in PBS buffer containing 0.1% DM at 4°C overnight with gentle agitation. The resin was washed two times with 200µl buffer (0.1% DM) and centrifuged at 10,000g for 2 minutes. After centrifugation, the supernatant were collected and spectra were measured.

In the case of Bio-MBP, the amount of protein in the supernatant fractions was measured by the method of Bradford using BSA as the standard.

2.2.8 Purification of Biotinylated Proteins

Purification of Biotinylated Proteins was achieved using Soft Link™ Soft Release Avidin Resin following the instructions of the manufacturer. The Soft Link Avidin Resin is a rigid, methacrylate polymeric gel filtration matrix, functionalized with covalently bound, monomeric avidin. Monomeric avidin binds biotin with a K_D of 10^{-7}, allowing reversible binding of bound biotinylated proteins under mild elution conditions. After the biotinylation reaction, free biotin was removed by dialysis against equilibration buffer. Samples were loaded onto a 1-ml column and washed with equilibration Buffer. The unbiotinylated protein was in the flow-through, the biotinylated protein was eluted from the resin with 5 mM biotin in equlibration buffer.

2.2.9 Assays by UV-visible Spectroscopy

Spectra of rhodopsin were obtained from 100 µl samples with BTP buffer used to set the baseline. Samples were measured first in the dark. Thereafter, samples were illuminated

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for 10 s using a fiber optic light source equipped with a > 480-nm longpass and a heat protection filter. Spectra of the photoproduct metarhodopsin II (λ_{max} = 380 nm) was recorded and the concentration of rhodopsin was determined spectrophotometrically using ε_{500} = 42,700 mol^{-1} cm^{-1} for all pigments.

2.2.10 Fluorescence Spectroscopy

Purified biotinylated rhodopsin (2 nM) was mixed with 250 nM Gt, in buffer (20 mM BTP, pH 7.5, 130 mM NaCl,1 mM $MgCl_2$ and 0.01% DM). The samples were mixed in the dark, and Gt activation was initiated by injection of GTPγs to a final concentration of 5 µM. Traces of Gt activation are a percent change of fluorescence emission at 345 nm recorded after exciting the sample at 300 nm.

2.2.11 The IAsys Technology

IAsys is based on a new optical evanescent sensor, called the resonance mirror. The resonant mirror is a simple structure of two dielectric layers on glass, which leads to a reproducible and robust performance. The device consists of a high refractive index wave guide separated from a high refractive index prism block by an intervening, low index coupling layer; see Fig. 7.

Laser light is directed at the prism over a range of angles (only one angle is shown for clarity). At all angles total internal reflection occurs from the prism coupling layer interface. At one unique angle, the resonant angle, light tunnels through the coupling layer and propagates in the resonant layer, and the tunneling process is reversible allowing some light to escape. Phase-detection is employed to isolate only that component of the light which propagates in the resonant layer.

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The evanescent field of the light propagating within the resonant layer encounters the sample immobilized on its surface. The intensity of the evanescent field decays exponentially and has reduced to one third of its intensity at the surface within a few hundred nanometers. It is the rapidly decaying envelope of the field intensity that ensures that only interactions of immobilized species are monitored.

Material entering the evanescent field from the sample will alter the refractive index profile close to the surface of the device, changing the resonance angle. The changing resonance angle is sampled approximately three times a second. The change in position of intensity peak can be precisely followed with sampling intervals of less than one second.

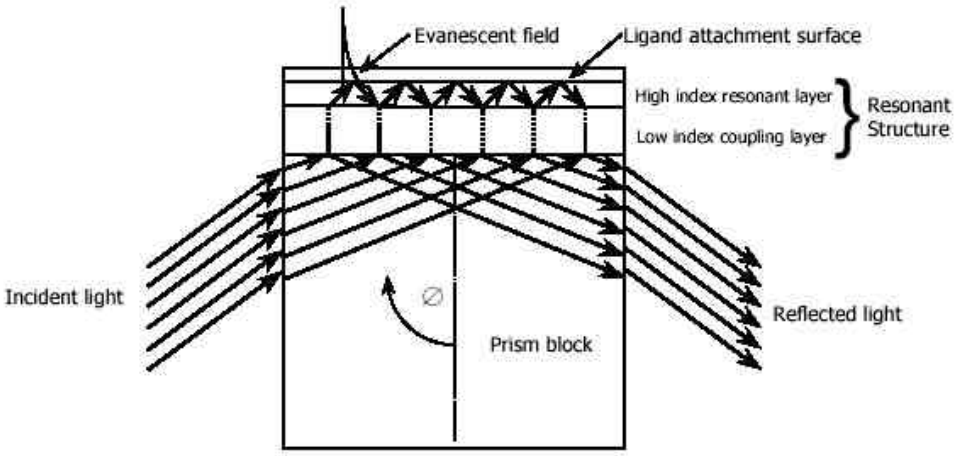

Fig.7 Configuration of the Resonant Mirror Sensor Device (www.Affinity-sensors.com)
Changes in refractive index at the ligand attachment surface of the device (the biological layer) change the angle at which light can be made to propagate in the waveguide. Laser light (670nm) is scanned at a 10° angle across the device, see Fig. 8.

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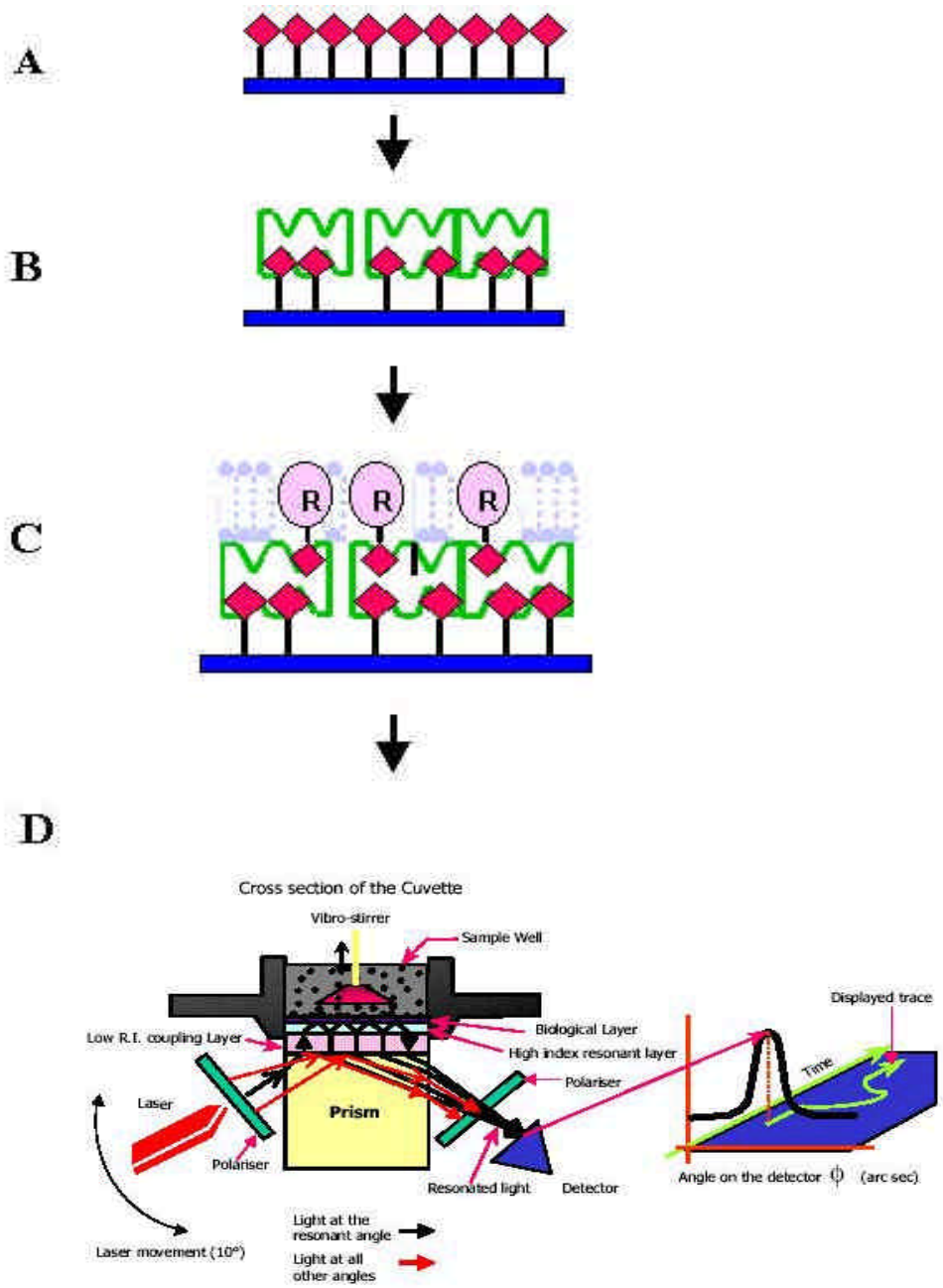

Fig. 8 The Procedure for IAsys Measurement A) the sensor chip of IAsys is modified with biotin molecules. B) NeutrAvidin binding to biotin. Free binding sites remain for binding of biotinylated rhodopsin. C) immobilization of biotinylated rhodopsin followed by formation of a supported lipid bilayer. D) Association or dissociation of the material immobilized on the sensor chip can be monitored by the IAsys system.

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The work on IAsys includes immobilization of rhodopsin onto the sensor chip, reconstitution of the immobilized rhodopsin into a lipid bilayer and measurement of Gt activation. (Fig. 8)

2. 2. 11. 1 Formation of Monolayer of Linker

The IAsys CUV991200 cuvette contains a modified sensor chip which is covered with biotin. The avidin/streptavidin was used as the traditional bridge between biotin and the biotin-tagged protein. For ultra-low nonspecific binding compared to Avidin or Streptavidin, NeutrAvidin^{TM} Biotin-binding protein (Pierce) was used as linker in our experiments. NeutrAvidin is a deglycosylated form of avidin, so lectin binding is reduced to undetectable levels without losing biotin binding affinity ($K_a=10^{15}$ M^{-1}). NeutrAvidin offers the advantage of a neutral pI to minimize nonspecific absorption, along with lysine residues that remain available for derivatization or conjugation through amine-reactive chemistries. The molecular weight of NeutrAvidin is approximately 60,000. The specific activity for biotin binding is approximately 14 µg/mg of protein, which is near the theoretical maximum activity.

Measurements were performed in stirred cuvettes (200 µl). After insertion of a new biotin cuvette into the IAsys device, the cuvette was washed three times with 200 µl of G1 buffer and allowed to equilibrate for 10 minutes. Then baseline data was collected for 3 minutes. Then NeutrAvidin was injected into the cuvette to a final concentration of 200 nM and incubated for 5 minutes, allowing it to bind to biotin in order to form linker layer of NeutrAvidin.

2. 2. 11. 2 Immobilization of Rhodopsin

Purified biotinylated rhodopsin (Rho-Bio) was injected into the cuvette to the final concentration of 1 µM and allowed to bind to the NeutrAvidin for 10 minutes.

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2. 2. 11. 3 Formation of A Supported Lipid Bilayer

After immobilization of rhodopsin, the cuvette was washed twice with 200 µl of OG 50 containing 1mg/ml egg-PC. To form supported lipid bilayer on the sensor surface, the micelle dilution technique (Lang et al., 1994) was applied. In micellar dilution, a detergent/lipid mixture is diluted with aqueous buffer below the critical micellar concentration of the detergent. This method is commonly used to prepare liposomes and proteoliposomes. On solid support, micellar dilution has been used primarily to deposit lipid monolayers on hydrophobic surfaces or to produce tethered lipid bilayers using thiolipids.

The lipid/detergent solution was diluted with G buffer 3 × 200 µl by slow injection and constant stirring.

2. 2. 11. 4 Transducin Binding and Rhodopsin Activition

After membrane formation, Buffer G1 in the cuvette was exchanged with buffer G, containing transducin at a concentration of 4.5 µM. After 15 minutes incubation, allowing transducin bind to the membrane, a light illumination ($\lambda \geq 495$ nm, 20 sec) was used to activate rhodopsin. Thereafter, GTP was added at a final concentration of 20 µM to initiate GTP uptake by Gt .

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