Fluorescence Probes and Labels for Biomedical Applications

Ewald Terpetschnig
Classification:

- Intrinsic Fluorophores
- Extrinsic Fluorophores
Intrinsic Fluorophores

Naturally Occurring Fluorophores
Proteins: Naturally Occurring Fluorophores

Aromatic amino acids

Phenylalanine (Phe – F)
Ex/Em 260 nm/282 nm

Tyrosine (Tyr – Y)
Ex/Em 280 nm/303 nm

Tryptophan (Trp-W)
Ex/Em 280, 295 nm/305-350 nm

Low Q.Y. Insensitive to solvent polarity

Insensitive to solvent polarity

Relative Intensity [a.u.]

Wavelength [n.m.]
Fluorescence Lifetimes of Protein-Related Fluorophores

**Tyrosine:**
- \( \lambda_{\text{Ex}} : 280\text{-nm LED} \\
  \text{Em} : 320\text{-nm LP} \\
  \tau = 3.2 \text{ ns (Water)}

**NATA:**
- \( \lambda_{\text{Ex}} : 300\text{-nm LED} \\
  \text{Em} : 320\text{-nm LP} \\
  \tau = 3.09 \text{ ns (Water)}
**Naturally Occurring Fluorophores**

**Enzyme Cofactors**

- **NADH**
  - (Oxido-reductases)
  - Ex/Em 340/460 nm

- **FAD**
  - (Metabolic enzymes)
  - Ex/Em 450nm/540 nm

**Porphyrins**
- Ex/Em 550 nm/620 nm

- Fe$^{2+}$ (Heme)
  - myoglobin,
  - hemoglobin
  - cytochromes b and c,
  - cytochrome P450 and
  - cytochrome oxidase

- Mg$^{2+}$ chlorophylls
Extrinsic Fluorophores

Synthetic dyes or modified biochemicals that are added to a specimen to produce fluorescence with specific spectral properties.
Fluorescent Probes:

- Non covalent interaction
  A fluorescent probe is a fluorophore designed to localize within a specific region of a biological specimen or to respond to a specific analyte.

Fluorescent Labels:

- Covalent interaction
Classes of Probes, Dyes and Labels:

- Organic Dyes
- Metal-Ligand Complexes
- Quantum Dots and Nanoparticles
- Fluorescent Beads or Polymers
- Fluorescent Proteins
Fluorescent Probes

Non-covalent

1,8-ANS

Developed by G. Weber in 1950’s

Barely fluorescent water - fluorescence is strongly enhanced in hydrophobic environments

Valuable probes for studying membranes and proteins: QY ~ 0.25 (membranes) or ~0.7 (proteins)
Fluorescent Probes

Albumin Blue

Measurement of albumin levels in biological samples including serum and urine.

Developed by Kessler & Wolfbeis

Specific- quantitative determination of Albumin in presence of other proteins

Albumin-specific also at concentrations below 100mg.L\(^{-1}\) - Microalbuminuria
Fluorescent Ion-Probes
Fluorescent Ion-Probes

Fluorescence probes have been developed for a wide range of ions:

Cations:
H+, Ca2+, Li+, Na+, K+, Mg2+, Zn2+, Pb2+ and others

Anions:
Cl−, PO₄²⁻, Citrate, ATP, and others
How to choose the correct fluorescent probe

**Dissociation Constant (Kd)**
- Must be compatible with the concentration (pH) range of interest.
- Calibration: Kd of probe is dependent on pH, temperature, viscosity, ionic strength etc.

**Measurement Mode**
Qualitative or quantitative measurements.
- Ion-probes showing spectral shifts - radiometric measurements
- Light source available

**Indicator Form**
Influences cell loading and distribution of the probe.
- Salts and dextran-conjugates - microinjection, electroporation
- AM-esters - passively loaded and cleaved by intracellular esterases
### pH-Probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>pH Range</th>
<th>Measurement Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNARF indicators</td>
<td>6.0-8.0</td>
<td>Em. ratio 580/640 nm</td>
</tr>
<tr>
<td>HPTS (pyranine)</td>
<td>7.0-8.0</td>
<td>Exc. ratio 450/405 nm</td>
</tr>
<tr>
<td>BCECF</td>
<td>6.5-7.5</td>
<td>Exc. ratio 490/440 nm</td>
</tr>
<tr>
<td>Fluoresceins and Carboxyfluoresceins</td>
<td>6.0-7.2</td>
<td>Exc. ratio 490/450 nm</td>
</tr>
<tr>
<td>Oregon Green dyes</td>
<td>4.2-5.7</td>
<td>Exc. ratio 510/450 nm</td>
</tr>
<tr>
<td>LysoSensor Yellow/Blue DND-160</td>
<td>3.5-6.0</td>
<td>Em. ratio 450/510 nm</td>
</tr>
</tbody>
</table>

Molecular Probes' pH indicator families, in order of decreasing $pK_a$. 
BCECF

R. Tsien 1982

Most widely used fluorescent indicator for intercellular pH

Membrane-permeant AM: pKa ~ 6.98 is ideal for intracellular pH measurements

Excitation-ratiometric probe with Ip at 439 nm, which is used a the reference point
Calcium-Probes

BAPTA

Chelator with high selectivity for Ca\(^{2+}\) in presence of excess [mM] Mg\(^{2+}\)

\(K_d\) for Ca\(^{2+}\) No Mg\(^{2+}\): 160 nM
1 mM Mg\(^{2+}\): 700 nM
Calcium-Probes

**UV**
- **FURA** (Fura-2, Fura-4F, Fura-5F, Fura-6F, Fura-FF)
- **INDO** (Indo-1, Indo 5F)

**VISIBLE**
- **FLUO** (Fluo-3, Fluo-4, Fluo5F, Fluo-5N, Fluo-4N)
- **RHOD** (Rhod-2, Rhod-FF, Rhod-5N)
- Calcium Green, Calcium Orange, Calcium Crimson
- Oregon Green 488-BAPTA

**Ratiometric**

**Non-Ratiometric**
Most used in conventional microscopic imaging

Good excitation shift with Ca$^{2+}$

Ratioed between 340 and 350 and 380 to 385 nm

Fura-4F, Fura-5F, Fura-6F and Fura-FF provide increased sensitivity to intracellular Ca$^{2+}$ concentration in the 0.5–35 µM range
**Indo-1**  
Emission-Ratiometric

**Indo-1** is most used in laser flow cytometry. It is ratioed between 450 and 405 nm. Photobleaches faster than Fura-2. Excitation is with UV laser or Ti-Sapphire at 350 nm.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>$K_d(Ca^{2+})$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>indo-1</td>
<td>0.23</td>
</tr>
<tr>
<td>indo-5F</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Fluorescence emission

Ex = 355 nm
Calcium Green-5N

Non-Ratiometric

Low affinity Ca-probe
Low fluorescence in absence of Ca$^{2+}$
Tracking rapid Ca$^{2+}$-release kinetics

Indicator | $K_d$(Ca$^{2+}$) |
--- | --- |
Calcium Green-5N | 14 uM |
Fluorescent Labels

[Chemical structure image]
Labeling should not alter the biological activity of biomolecules.

**Selecting the Label**

PROTEIN → Reactive Group → Reactive Group → Fl. Label

- Reactive groups on proteins:
  - $\text{NH}_2$: Lysine N-terminus
  - $\text{SH}$: Cysteine

- Depends on the reactive group on the protein

- Light source
- Spectral properties
- Autofluorescence
- Photostability

Labeling should not alter the biological activity of biomolecules.
Protein Labeling

Amino-Modification:

- Isothiocyanate:
  \[ \text{Fl}-\text{N}=\text{C}=\text{S} \rightarrow \text{Fl}-\text{NH}=-\text{C}-\text{NH}-\text{Protein} \]
  Thiourea

- Succinimidyl ester:
  \[ \text{Fl}-\text{C}=\text{O}=-\text{NO} \rightarrow \text{Fl}-\text{C}=\text{NH}-\text{Protein} \]
  Carboxamide

- Sulfonyl chloride:
  \[ \text{Fl}-\text{SO}_2\text{Cl} \rightarrow \text{Fl}-\text{SO}_2\text{NH}-\text{Protein} \]
  Sulfonamide

- Aldehyde:
  \[ \text{Fl}-\text{CH}=\text{N} \rightarrow \text{Fl}-\text{CH}=\text{N}-\text{Protein} \]
  Schiff Base

  Reduction

Protein-\(\text{NH}_2\)

Lysine N-terminus
Protein Labeling

Thiol-Modification:

Protein-SH + Cysteine

alkyl halides or iodoacetamides

Protein-SH

Maleimide

Thioether
Labeling Procedure

1. Protein in buffer

2. Incubation time

3. Add fluorescent dye
   dye/protein starting ratio

4. Removal of free dye

5. Sephadex Column Chromatography or Dialysis

Labeling Ratio

[dye] [protein]

Sample Characterization

- Absorption spectra
- Protein determination

Biological Testing

- Activity measurements
- SDS or native gel
- Immunoassay etc.
Determination of Dye-to-Protein Ratios

\[
D/P = \frac{A_{\text{conj}(\lambda_{\text{max}})} \cdot \varepsilon_{\text{Prot}}}{(A_{\text{conj}(280)} - x \cdot A_{\text{conj}(\lambda_{\text{max}})}) \cdot \varepsilon_{\text{dye}}}
\]

\[
x = \frac{A_{\text{dye}(280)}}{A_{\text{dye}(\lambda_{\text{max}})}}
\]

Protein:
- Photometric measurement
- Bradford, Lowry assay
Organic Dye Classes

Xanthenes
- Fluoresceins - pH-sensitive, not photostable
- Rhodamines - tendency to aggregate in aqueous solution

Coumarins - low extinction coefficients, phototoxic

BOPIDYs - non-polar and relatively insoluble in water

Phthalocyanaines - photostable but difficult to synthesize and purify

Cyanines - high extinction coefficients and reasonable quantum yields
Examples of Extrinsic Labels

- **FITC**
  
  (488/512), $\tau \approx 4.0$

- **Texas Red-NHS**
  
  (595-615), $\tau \approx 3.5$ ns

- **BODIPY**
  
  (493/503), $\tau = 6$ ns

- **IAEDANS**
  
  (360/480), $\tau \approx 15$ ns

- **Coumarin-3-carboxylic acid -NHS**
  
  (445/482), $\tau \approx 2$ - 3 ns

- **LaJolla Blue**
  
  (680/700)
Absorption of Biological Material

Absorption Coefficient [cm$^{-1}$]

Wavelength [nm]

- UV
- Vis
- IR

- whole blood
- melanosomes
- epidermis
- aorta
- skin
- Laser Diodes: 370, 405, 473, 532, 635, 670
- Nd: YAG
- Er: YAG
- CO$_2$

75% water
Spectral Properties and Quantum Yields of Representative Long-Wavelength Absorbing and Emitting Dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{max}}$ (abs) [nm]</th>
<th>$\lambda_{\text{max}}$ (em) [nm]</th>
<th>$\varepsilon$ (M(^{-1})cm(^{-1}))</th>
<th>Lifetime $\tau$ [ns]</th>
<th>Q.Y. (H(_2)O)</th>
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<tbody>
<tr>
<td>Cy5</td>
<td>649</td>
<td>670</td>
<td>250,000</td>
<td>1.0</td>
<td>0.3</td>
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<tr>
<td>Dy650</td>
<td>649</td>
<td>670</td>
<td>120,000</td>
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<tr>
<td>Alexa 647</td>
<td>647</td>
<td>666</td>
<td>265,000</td>
<td>1.0</td>
<td>0.33</td>
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<tr>
<td>DyLight 649</td>
<td>646</td>
<td>674</td>
<td>250,000</td>
<td>1.0</td>
<td>0.33</td>
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<tr>
<td>HiLyte 647</td>
<td>649</td>
<td>674</td>
<td>250,000</td>
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<tr>
<td>BODIPY</td>
<td>629</td>
<td>646</td>
<td>97,000</td>
<td>3.9</td>
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<td>Atto</td>
<td>661</td>
<td>678</td>
<td>100,000</td>
<td>1.8</td>
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HPLC-Retention Times of Red Fluorescent Dyes

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<tr>
<th>Hydrophobicity</th>
<th>Alexa 647</th>
<th>ATTO 655</th>
<th>ATTO 680</th>
<th>Bodipy 630/650</th>
<th>Cy 5</th>
<th>Cy 5.5</th>
<th>Dy-630</th>
<th>Dy-635</th>
<th>Dy-640</th>
<th>Dy-650</th>
<th>Dy-655</th>
<th>EVOblue30</th>
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<td>Retention time (min)</td>
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Structure and Spectral Properties of Long-Wavelength Cy Dyes™

<table>
<thead>
<tr>
<th>Dye-Conjugate</th>
<th>( \lambda_{\text{max (abs)}} ) [nm]</th>
<th>( \lambda_{\text{max (em)}} ) [nm]</th>
<th>( \varepsilon ) (M(^{-1})cm(^{-1}))</th>
<th>Q.Y.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5</td>
<td>649</td>
<td>670</td>
<td>250,000</td>
<td>0.2-0.28</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>675</td>
<td>694</td>
<td>250,000</td>
<td>0.28</td>
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<tr>
<td>Cy7</td>
<td>743</td>
<td>767</td>
<td>250,000</td>
<td>0.28</td>
</tr>
</tbody>
</table>
Structure and Spectral Properties of Long-Wavelength ALEXA Dyes™

<table>
<thead>
<tr>
<th>Dye-Conjugate</th>
<th>$\lambda_{\text{max (abs)}}$ [nm]</th>
<th>$\lambda_{\text{max (em)}}$ [nm]</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>Q.Y.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF647</td>
<td>650</td>
<td>668</td>
<td>250,000</td>
<td>0.33</td>
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<tr>
<td>AF660</td>
<td>668</td>
<td>668</td>
<td>132,000</td>
<td>0.37</td>
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<tr>
<td>AF750</td>
<td>749</td>
<td>782</td>
<td>240,000</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Quantum Yields and Total Fluorescence of IgG-Conjugates of Cy5 (▼), and Alexa-647(■) for Various D/P Ratios

Cy5 has anomalous tendency to aggregate
Cy5 - Anomalous Tendency to Aggregate

1\textsuperscript{st} Cy5 molecule associates with the surface of a protein and reacts

2\textsuperscript{nd} molecule interacts with labeled Cy5 and then reacts with next closest amino-group

Time-Resolved Luminescence Measurement

![Graph showing time-resolved luminescence measurement](image)
Luminescent Lanthanides (Eu$^{3+}$, Tb$^{3+}$)

Advantages vs. Organic Fluorophores:

Enable Discrimination of Short-Lived Autofluorescence by “Gating” - More Sensitive and Reliable Measurements

Narrow Emission Bands

Long Luminescent Lifetimes

Large Stokes’ Shifts
Homogeneous Time-Resolved Fluorescence Immunoassays (HTRF)

Minimal Sample Preparation - No Wash Steps

Applications: Drug Discovery

Clinical Diagnostics
What Is The Mechanism?

**Antenna Effect**

“Antenna Effect”: Strong Absorption and Good Energy Transfer

Ln(III) Protected from Quenching

Thermodynamically Stable and Kinetically Inert Complexes
Lanthanide Complexes for Homogeneous Time-Resolved Immunoassays

**LANCE Ultra™**

- Eu$^{3+}$ - Luminescence
- $\phi = 11 - 15 \%$, $\lambda_{\text{excitation}} \sim 320$ nm

- CH- and CH$_2$ -Group are Replaced by CD and CD$_2$

- Acceptors: Allophycocyanine (APC) or cyanine dyes
Lanthanide Complexes for Homogeneous Time-Resolved Immunoassays

Eu-Cryptate™

Luminescent Only in Presence of F⁻

φ = 2 %

Not Stable in Water but “Kinetically Inert”

1-3 Water Molecules are Coordinated to Ln(III) (Limited Protection)

Acceptor : XL665 or C2 a low-MW acceptor
Highly Luminescent Lanthanide Complexes

2-hydroxy-isophthalamide ligands

Quantum Yield: 60 % !!!

Lifetime \( \tau = 2.56 \, \text{ms} \)

Lumiphore, Inc.
TR-FRET HTS Assay

384-well Plate

5 µl enzyme

Incubation at RT for 1h

+ 5 µl ULight-peptide (+ ATP)

Incubation at RT for 1h

+ 10 µl Eu-anti-phosphosubstrate antibody (+ EDTA)

Read

(Excitation at 320 nm, Emission at 665 nm)

Courtesy of PerkinElmer
Spectral Data and Lifetimes for a Representative Metal-Ligand Complexes (MLCs)

**Ru(bpy)$_2$(dcbpy)**
- $\lambda_{\text{max}}(\text{abs})$ (water) = 467 nm
- $\lambda_{\text{max}}(\text{em})$ = 655 nm
- Q.Y. = 0.05
- $r_0$ = 0.23
- $\tau$ = 366 ns

**Ru(SO$_3$dphephen)$_2$(dcbpy)**
- $\lambda_{\text{max}}(\text{abs})$ (water) = 4640 nm
- $\lambda_{\text{max}}(\text{em})$ = 643 nm
- Q.Y. = 0.06
- $r_0$ = 0.8 µs
- $\tau$ = 2 µs (HSA)

**Re(CO)$_3$Cl(phen)**
- $\lambda_{\text{max}}(\text{abs})$ (water) = 275 nm
- $\lambda_{\text{max}}(\text{em})$ = 589 nm
- Q.Y. = 0.2
- $r_0$ = 0.3
- $\tau$ = 110 ns
Excitation Polarization Spectra of Representative MLCs
Fluorescence Polarization: A Race between Emission and Molecular Motion

Polarized light excites molecules aligned with plane of polarization.

$\tau_{fl} \ll \theta_{rot}$

Excited molecules remain aligned. Fluorescence is polarized.

$\tau_{fl} \gg \theta_{rot}$

Orientation of excited molecules randomizes. Fluorescence is depolarized.
Fluorescence Polarization Measurement
Fluorescence Polarization

Polarization \( (P) \) = \( \frac{I_v - I_h}{I_v + I_h} \)

Anisotropy \( (r) \) = \( \frac{I_v - I_h}{I_v + 2 I_h} \)

\[
P = \frac{3r}{2} + r
\]

\[
r = \frac{2P}{3} - P
\]
Role of Lifetime in FP

\[ \tau_{fl} \approx 300 \text{ ns} \]

\[ \theta = 150 \text{ ns} \]

\[ \tau_{fl} = 4 \text{ ns} \]
Quantum Dots

- CdSe Core
- CdSe/ZnS Core-Shell

- Cd
- Se
- Zn
- S
Cadmium selenide (CdSe), or Cadmium telluride (CdTe) few hundred – few thousand atoms

The semiconductor material is chosen based upon the emission wavelength, however it is the size of the particles that tunes the emission wavelength.

In the core emission is typically weak and always unstable. The shell material (ZnS) has been selected to be almost entirely unreactive and completely insulating for the core.

A layer of organic ligands covalently attached to the surface of the shell. This coating provides a surface for conjugation to biological (antibodies, streptavidin, lectins, nucleic acids) and nonbiological species and makes them “water-soluble”
Bandgap of nanocrystal is size-dependent, larger than for bulk material.
Preventing Photobleaching In Quantum Dots

Plain core QDots show emission, but oxidation results in permanent loss of emission, so the population would gradually bleach away.

In Core/shell QDots the electron remains in the lower-energy core orbitals, and never reaches particle surface to react.

The shell keeps the high-energy excited electron away from oxygen.
Semiconductor Nanocrystal Fluorescence

A high-energy photon excites an electron across the bandgap

A bandgap-energy photon is emitted as the electron falls back to the ground state

Size-dependent bandgap means size-dependent color

Courtesy of Invitrogen
Quantum Dots
Nanometer-Scale Atom Clusters

<table>
<thead>
<tr>
<th>Quantum Dot Material System</th>
<th>Emission Range</th>
<th>Quantum Dot Diameter Range</th>
<th>Quantum Dot Type</th>
<th>Standard Solvents</th>
<th>Example Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>CdSe</td>
<td>465nm - 640nm</td>
<td>1.9nm - 6.7nm</td>
<td>Core</td>
<td>Toluene</td>
<td>Research, Solar Cells, LEDs</td>
</tr>
<tr>
<td>CdSe/ZnS</td>
<td>490nm - 620nm</td>
<td>2.9nm - 6.1nm</td>
<td>Core-Shell</td>
<td>Toluene</td>
<td>Visible Fluorescence Applications, Electroluminescence, LEDs</td>
</tr>
<tr>
<td>CdTe/CdS</td>
<td>620nm - 680nm</td>
<td>3.7nm - 4.8nm</td>
<td>Core-Shell</td>
<td>Toluene</td>
<td>Deep Red Fluorescence Apps.</td>
</tr>
</tbody>
</table>
Qdot Optical Spectra

Violet excitation

Absorbance $\times$ Quantum Yield = Brightness
photons in fraction converted photons out

High absorbance means increased brightness
Single-color excitation, multicolor emission for easy multiplexing

Courtesy of Invitrogen
Brightness Means Sensitivity

**Qdot® nanocrystals**
- 0.019 s exposure

**Cy5 organic dye**
- 1.22 s exposure

**Anti-Her2/neu + anti-mouse Ig conjugates**
- SK-BR-3 Cells: High Her2/neu expression
- Nanocrystals up to 50x brighter

**MDA-MB-231 cells: Low Her2/neu expression**
- Nanocrystals easy to detect but dye undetectable

Courtesy of Invitrogen
Photostability in Microscopy

3T3 Cells

Top panel (a-e)
Nucleus: Qdot® 605 conjugate
Microtubules: Alexa Fluor® 488 conjugate

Bottom panel (f-j)
Nucleus: Alexa Fluor® 488 conjugate
Microtubules: Qdot® 605 conjugate

Photostability results in sensitivity, ease of use, and sample permanence

Courtesy of Invitrogen
**Qdot Summary**

**Advantages:**
- Broad absorption spectra, making it possible to excite all colors of QDs simultaneously with a single light source - **Multiplexing**
- Narrow and symmetrical emission spectra
- **Emission tunable** with size and material composition
- Exhibit excellent **photo-stability**

**Disadvantages:**
- **Large size and high mass** limit their use in applications requiring high diffusional mobility

<table>
<thead>
<tr>
<th>QDot</th>
<th>$\lambda_{\text{max (abs)}}$ [nm]</th>
<th>$\lambda_{\text{max (em)}}$ [nm]</th>
<th>$E$ (M$^3$cm$^{-1}$)</th>
<th>Q.Y.</th>
</tr>
</thead>
<tbody>
<tr>
<td>655</td>
<td>350</td>
<td>655</td>
<td>9,000,000</td>
<td>~0.5</td>
</tr>
<tr>
<td>705</td>
<td>350</td>
<td>705</td>
<td>13,000,000</td>
<td>~0.5</td>
</tr>
<tr>
<td>800</td>
<td>350</td>
<td>800</td>
<td>13,000,000</td>
<td>~0.5</td>
</tr>
</tbody>
</table>
Thank You